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Feeding experiments on substitution of protein by definite mixtures of isolated amino acids.

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FEEDING EXPERIMENTS ON THE SUBSTITUTION OF PROTEIN BY DEFINITE MIXTURES OF ISOLATED AMINO ACIDS

BY

HAROLD HANSON MITCHELL

A. B., University of Illinois, 1909

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FEEDING EXPERIMENTS ON THE SUBSTITUTION OF PROTEIN BY DEFINITE MIXTURES OF ISOLATED AMINO ACIDS

I. INTRODUCTION

The comparatively recent far-reaching researches on the chemistry of the proteins, inaugurated by Kossel and greatly extended by Fischer, have been of great importance to nutrition, and more specifically to the study of protein metabolism. While it has been known since the time of Liebig that amino acids could be isolated from the products of the hydrolytic decomposition of proteins, the real significance of this fact to the chemistry of the proteins, the identification of the various amino acids found in combination in proteins, and even the demonstration of the nature of this combination, we owe to the above-mentioned investigators, and especially to Emil Fischer and his associates. The extensive work of Abderhalden, and of Osborne, and more recently of Van Slyke, in this country has also added immensely to our knowledge of protein chemistry, until today, tho the difficulties to be overcome are still numerous, the problem is not by any means as hopeless as it has long been regarded. Indeed, it has well been said that if some of the more complex polypeptides synthesized in Fischer's laboratory had been found in nature, they would very probably have been regarded as proteins and have been duly classified with them.

Of still greater significance to the problems of protein metabolism than these purely chemical inroads into the subject are the more recent demonstrations that the natural digestion of proteins in the alimentary tract follows in general the same lines as their hydrolytic cleavage in the laboratory, especially the cleavage brought about by acid hydrolysis. The ability of the proteolytic enzymes of the stomach and intestines to degrade proteins completely to the simpler polypeptides and the amino acids, the isolation and identification of amino acids thruout the length of the small intestine during protein digestion, and the discovery by Cohnheim of an energetic proteolytic enzyme, erepsin, in the intes-

tinal walls themselves, have completed the demonstration that the amino acids are the Bausteine of the proteins just as dextrose is of starch. The researches of Folin and Denis and of Van Slyke and Meyer, completed within the last two or three years, have confirmed preceding investigations and have greatly extended their significance by showing that protein material is absorbed into the blood and ultimately reaches all the tissues of the body largely if not entirely in the form of amino acids.

The natural and inevitable consequences of these recent acquisitions to protein chemistry, protein digestion, and protein metabolism have been, first, to complicate immensely the problems of the nutritive value of proteins and of their fate in metabolism. It has not been very long since the nutritive value of a food, diet, or ration was supposed to be completely defined by its content of protein, carbohydrate, and fat, nor since the question of protein requirements and the protein minimum, irrespective of the nature of the protein itself, engrossed the attention of many physiologists and dietitians. In those days one protein was supposed to be just about as valuable to the body as another, with the possible exception of gelatin, the inadequacy of which for the maintenance of life had been known for many years. Now we are aware of the fact that proteins differ, and may differ markedly, as regards their value in nutrition, such differences being due to differences in their amino acid content. Seventeen amino acids are known to have a rather wide distribution among the different proteins, while two or three others have been identified in isolated instances. Furthermore, the possibility that many other amino acids are widely distributed in proteins is not precluded by the incomplete protein analyses obtained with the Fischer esterification method or the recent method of Van Slyke; in fact, this possibility must be conceded a considerable probability.

And thus the second consequence of the new view point necessitated by recent work, more particularly on the digestion and absorption of proteins, has been to shift the emphasis from the protein molecule itself, to the constituent amino acids to which it is reduced, apparently completely, in the digestive tract. The inves-

tigator in animal nutrition today instead of being confronted with the problems of the precise nutritive importance of protein as a single food nutrient, must consider the possibility of each of the many amino acids occurring naturally in proteins possessing specific and distinctive functions in metabolism, and as regards some of these amino acids he must face the certainty that such is the case. The protein requirements for maintenance, growth, and muscular work must now be analyzed into the requirements of each one of a series of widely distributed amino acids.

Investigations concerned with the question of the indispensability or otherwise of amino acids in nutrition, and with their precise significance, have been pursued notably along two general lines. The first line of investigation, altho followed by several workers independently with varying results, has been perfected by Abderhalden and has given the most fruitful results in his hands.

It has been repeatedly proven experimentally that the animal organism can be maintained in equilibrium and can even exhibit considerable increases in weight when the nitrogenous requirements are covered by preparations obtained by hydrolyzing protein material with enzymes until no positive biuret test could be obtained, when presumably only amino acids and perhaps the simpler polypeptides were present. Such experiments have been performed mostly upon dogs, the evidence that the human organism possesses the same capacity has also been obtained.[1]

[1] Abderhalden, Frank, and Schittenhelm: Zeitsch. physiol. Chem., 63, p. 215, 1909.

The most successful experiment thus far reported is that of Abderhalden[2] in which

[2] Zeitsch. physiol. Chem., 83, pp. 444-457, 1913.

a dog was kept for 100 days on a completely hydrolyzed meat preparation. During this time the animal behaved normally, seemed in perfect health, and increased in weight from 13.75 kgs. to 23.10 kgs.

On the basis of such results, the essence of Abderhalden's method was to re-

move certain amino acids from the unknown mixture of the products of protein hydrolysis and to observe the effects. Thus, the removal of tyrosine from the hydrolysis mixture by concentration, rendered it no longer capable of covering the nitrogen requirements or of maintaining the body weight of a dog. Similarly, the removal of tryptophane, or more accurately its reduction to mere traces, had a distinct and almost immediate deleterious effect, not so much on the body weight as on the behavior of the animal. Upon adding the particular amino acid, tyrosine or tryptophane, again to the amino acid mixture, the dog increased in weight and its behavior became normal. From these and other experiments Abderhalden concludes that tryptophane and tyrosine are both indispensable. The observation that the lack of tryptophane from the ration quickly led to the appearance of severe symptoms, i.e., lethargy, a marked tendency to tire easily, etc., made it appear probable to Abderhalden that Hopkins is correct in assuming that this amino acid is the starting point for the synthesis of the characteristic constituents of various internal secretions. This work on the indispensability of tyrosine and tryptophane has been repeatedly confirmed.

Abderhalden also attempted[1] to determine the importance of proline, by com-

[1] Zeitsch. physiol. Chem., 77, p. 50, 1912.

paring the results of a seven-day feeding period on a dog, during which a complete hydrolysis mixture was fed, to an immediately following six-day period during which a hydrolysis mixture containing no proline (extracted with absolute alcohol) was fed. Upon observing no marked variations in body weight or in nitrogen equilibrium in the latter period, he concluded that proline may be synthesized from some other amino acid, and is therefore not indispensable to the body. Upon comparing the daily nitrogen balances of the second period with those of the first, and also with those of the succeeding period, during which a complete hydrolysis mixture was fed, it may be seen that they are in general less than those of the other two periods. Aside from this criticism, however, there is the more obvious one pertain-

ing to the short duration of the experimental periods. The conclusion is favored, however, by certain theoretical considerations. Thus, the relation in structure of proline, ornithine, and glutamic acid is suggestive of a possible interconversion.

Abderhalden has also endeavored[1] to determine whether cystine is indispensable

[1] Zeitsch. physiol. Chem., 83, p. 445, 1913

to the animal organism or whether it can be replaced by other sulfur-containing compounds, such as the inorganic sulfates. The growth of hair on a dog during feeding with a complete mixture of amino acids was compared with the growth of hair during feeding with the same mixture from which the cystine had been withdrawn. The results of the experiment, however, were not susceptible to a definite interpretation.

Henriques and Hansen,[2] experimenting on rats, were unable to obtain maintenance

[2] Zeitsch. physiol. Chem., 43, pp. 417-446, 1904

of body weight or positive nitrogen balances on synthetic rations containing the products of the complete acid (HCl and H_2SO_4) hydrolysis of casein as the sole nitrogenous constituents. In several short experiments (18 to 20 days) more successful results were obtained with the products of enzyme hydrolysis of proteins. Slight increases in body weight were recorded, but the nitrogen balances were in general unsatisfactory, especially when it is considered that the determination of the total nitrogen in the urine of rats, with the exercise of the utmost care, has been shown by Osborne and Mendel[3] to be subject to errors of 10 percent or more, due

[3] Carnegie Institution Publication No. 156, Part I, p. 12, 1911

to incomplete collection.

The most interesting experiment of these investigators and the one most pertinent to the present discussion involved feeding with a mixture containing presumably only monoamino acids. It was prepared from an enzyme digest of a protein by precipitation with phosphotungstic acid, filtration, removal of phosphotungstic

acid from the filtrate, and evaporation to dryness in vacuo. Unfortunately the particular protein used was not mentioned, and the lack of all details of the phosphotungstic acid precipitation precludes any attempt to judge of the completeness of the separation of monoamino from diamino acids. The ration of the experiment in question contained 80 g. of fat, 45 g. of monoamino acids prepared as above described, 10 g. of sugar, 20 g. of cellulose and 4 g. of salts, the nitrogen content being 2.79 percent. The experiment lasted 26 days. During the last 17 days only were positive nitrogen balances obtained and they were such that they could hardly be said to constitute convincing evidence of the nutritive adequacy of the ration fed. The body weight increased slightly during the last half of the experiment, but during the last three days it declined slightly but consistently. It is unfortunate that the experiment was not continued further to determine whether this final decrease was significant or not. On the whole, it cannot be said without further experimentation, that the diamino acids are actually dispensable for the maintenance of body equilibrium.

The second general method of investigation of the nutritive significance of the amino acids to the animal organism is associated more especially with the names of Osborne and Mendel and their associates, and has given by far the most valuable results in their hands. The method consists essentially in the feeding of isolated and carefully purified proteins, obtained from a wide variety of sources, to basal rations otherwise adequate for normal growth or maintenance, composed of artificial food materials, in most part of known composition and containing at most but minimal percentages of nitrogen or protein. By this method, different proteins have been clearly shown to have different values in nutrition, in one respect or another, and these differences have been traced with great success to differences in amino acid content. Thus, the significance of some of the amino acids have been made evident, either by noting the peculiar effect attendant upon their absence in proteins or their occurrence in minute amounts, or by observing the effects produced upon supplementing proteins by their addition.

The most valuable and significant of the many researches of Osborne and Mendel performed exclusively on white albino rats, will be briefly mentioned. Of perhaps the most general biological significance is the demonstration that the amino acid requirements for growth are apparently qualitatively different from those for maintenance. Thus, casein, containing all of the amino acids ordinarily isolated from proteins with the exception of glycocoll, is capable when ingested in sufficient amounts of supporting normal growth as well as mere maintenance. Gliadin, the alcohol-soluble protein of wheat, on the other hand, is capable of maintaining an animal for long periods of time at constant weight but is not able to support normal growth. A slow growth was sometimes secured, while at other times only maintenance resulted. This characteristic stunting with gliadin results even when the calorie intake is sufficient for optimum growth, and has been successfully traced[1]

[1] Journ. Biol. Chem., 17, No. 3, p. 332, April 1914

to the almost total absence of the diamino acid lysine from this protein. Thus, upon supplementing gliadin with lysine normal growth was secured.

Again, zein, the alcohol-soluble protein of the corn kernel, has been shown by Osborne and Mendel (and others before them) to be unable to support either growth or maintenance. Upon analysis this protein has been shown to lack the amino acids glycocoll, tryptophane, and lysine. The addition of tryptophane to this protein renders it adequate for long continued maintenance.[2] The further

[2] Journ. Biol. Chem., 20, No. 3, p. 378, March 1915.

addition of lysine renders it adequate for fairly rapid growth,[2] but for normal growth a further addition of arginine, which is present only in small amount in zein, appears[3] to be necessary.

[3] Journ. Biol. Chem., 18, No. 1, p. 11, June 1914.

Similarly, the feeding of gelatin, a protein totally deficient in tyrosine

and tryptophane, is insufficient to prevent loss of weight, apparently regardless of the amount consumed. The attempt to supplement this protein with tyrosine and tryptophane and thus secure maintenance of body weight, has up to the present been unsuccessful for unknown reasons. An analogous case is presented by phaseolin, a globulin obtained from the white kidney bean. This protein is not entirely deficient in any of the amino acids commonly yielded by proteins, but when fed as the sole protein to rats[1] is unable to support either maintenance or growth.

[1] Journ. Biol. Chem., 18, No. 1, p. 9, June 1914.

This nutritive failure of phaseolin remains unexplained.

Recently, in an investigation[2] to determine the effect of different percentages of protein in the ration on the growth of white rats, Osborne and Mendel have shown that percentages of casein inadequate for normal growth, may be rendered adequate by the addition of the sulfur-containing amino acid, cystine, to the ration to an amount equal to 3 percent of the contained casein. With percentages of casein in the ration of 9 or less, marked improvement in growth was shown to follow additions of cystine. These results are intelligible in view of the comparatively low cystine content of casein.

[2] Journ. Biol. Chem., 20, No. 3, pp. 351-377, March 1915.

The above investigations confirm the work of Abderhalden in showing the indispensability of tryptophane for either maintenance or growth, and also establish clearly the necessity of lysine for growth, less certainly[3] the necessity of cystine for growth, and render probable, the necessity of arginine for growth. The latter point, however, calls for further investigation.

[3] Whether the favorable influence of cystine when added to diets containing 9 percent or less of casein, was due to its sulfur content entirely and could be duplicated by some other available sulfur-bearing compound, is not clear. It is to be noted that the ration as a whole, as well as the casein, was poor in sulfur.

The last publication of Osborne and Mendel cited is of further interest in

this connection in that it demonstrates clearly that the protein requirements for maintenance, in the rat at least, are extremely small and that the protein requirements for growth are much smaller than was previously supposed. Their ordinary rations in previous experiments, both for maintenance and growth, have contained 18 percent of protein. In this investigation, however, maintenance for long periods (170 days) was secured with rations containing only 2 percent of lactalbumin, and the possibility that this percent could be materially lowered by properly supplementing this protein with amino acids, is rendered extremely probable by the general trend of the results obtained by these investigators. Furthermore, normal growth was secured with rations containing 9 percent of lactalbumin, casein supplemented by cystine, and edestin supplemented by lysine. Again the possibility of lowering this percentage considerably must be conceded a fair probability. McCollum and Davis[1] have independently obtained similar results to those of Osborne

[1] Journ. Biol. Chem., 20, No. 3, pp. 415-428, March 1915.

and Mendel, securing maintenance of rats on rations containing 3 percent of milk protein and normal growth on rations containing 6 percent.

Thus, the requirements of the animal body for protein (if this generalization from experiments on rats is permissible) approaches the same order of magnitude as the requirements for the so-called "accessory substances", especially the protein requirements for mere maintenance of body equilibrium. These extremely interesting experimental findings confirm a theory previously advanced[2] by Osborne and Men-

[2] Journ. Biol. Chem., 17, No. 3, pp. 328-329, April 1914.

del, regarding the amino acid requirements for maintenance. Quoting these investigators:

"The remarkable fact that an animal can be maintained satisfactorily over very long periods, not merely a few days or weeks, on a diet which promptly becomes a promoter of growth by the mere substitution of another protein for gliadin, raises the question whether, after all, protein in the broader sense is necessarily destroyed by the so-called 'wear and tear' of the body.....It is quite conceivable that what has hitherto been called the 'wear and tear' (Abnutzungsquote) is in re-

ality the demand for an amino acid necessary for the construction of some non-protein nitrogenous substance essential for normal metabolism rather than a requirement for the replacement of all the 'Bausteine' which go to make up cellular proteins. On the basis of present evidence it is quite as logical to assume that the maintenance requirement is in reality a requirement for definite (as yet specifically undetermined) amino acids that serve special physiological functions, as it is to insist that protein as such is demanded to repair a hypothetical destruction of the entire protein molecule."

This theory that the protein requirement for maintenance is really a requirement for one, or at most a relatively few amino acids, for the replenishment of the organism's stock of certain non-protein substances of vital physiological importance -- such as, for instance, the active constituents of the secretions of the various ductless glands -- receives considerable support from experiments on the utilization of proteins inadequate for maintenance. According to the ordinarily accepted theories of protein metabolism, a protein inadequate for maintenance, such as zein or gelatin, that is, a protein totally deficient in one or more of the amino acids that are necessary for tissue construction and that the body cannot synthesize, would be of no use in metabolism except as a source of energy. The nitrogen would all appear immediately in the urine. However, McCollum has shown[1] that the

[1] Wis. Agr. Exp. Sta., Research Bul. 21.

pig "can utilize the nitrogen of zein very efficiently for repair of the losses due to endogenous or tissue metabolism. The average utilization of zein nitrogen for this purpose was about 80 percent, for gelatin 50 to 60 percent. No evidence was obtained of the formation of additional body tissue from zein, even when the latter was fed in great excess over the maintenance needs of the animals." In other words, assuming a constant endogenous metabolism, these percentages of the nitrogen of zein and gelatin were retained by the animals and apparently utilized for some purpose other than the construction of protein tissue. Similarly Henriques and Hansen[2] have shown that the protamine clupein (fed as the sulfate), a protein

[2] Zeitsch. physiol. Chem., 49, pp. 113-123, 1906

consisting of arginine to the extent of about 82-83 percent, was inadequate for

maintenance of the white rat, but that its nitrogen was, nevertheless, utilized to some extent in metabolism.

In properly appraising the two general lines of investigation of the metabolic significance of amino acids briefly described, on the one hand, that method associated mainly with the name of Abderhalden, and, on the other hand, that exemplified best by the work of Osborne and Mendel, it must be admitted that they have added immensely to our knowledge of the question. Our conceptions of protein metabolism must be profoundly remodeled as a result of this pioneer work. The work of Osborne and Mendel in particular is worthy of the highest commendation. By working with small experimental animals and using artificially prepared rations, consisting in large part of carefully purified substances of more or less definite composition, they have succeeded in performing numerous experiments under carefully controlled conditions. The results have been clear cut and unequivocal in most instances. However, no criticism of these methods would be complete without due account being taken of their limitations.

The limitations of the Abderhalden method are obvious. It is only applicable to those amino acids which can be readily and quantitatively isolated from an unknown amino acid mixture, and isolated by means of reagents which can subsequently be removed from the residual mixture. In view of the difficulty of such isolation in the case of the large majority of the naturally occurring amino acids, this method has apparently well-nigh exhausted itself in demonstrating the nutritive value of tyrosine and tryptophane. The effect of the removal of proline by extraction with absolute alcohol, and of the diamino acids by precipiation with phosphotungstic acid still needs confirmation. It is hardly conceivable in the present state of the methods for the quantitative separation of the amino acids, how this first method of Abderhalden's can be of any lasting value, without at the same time becoming so cumbersome as to be of little practical utility.

The limitations of the Osborne and Mendel method are also obvious. Its success depends upon a good knowledge of the amino acid content of the different pro-

teins investigated. The extremely unsatisfactory condition of this knowledge as regards the large majority of the proteins studied needs no comment. Its most striking results have been afforded by a study of the nutritive value of proteins totally deficient (or very nearly so) in one or more amino acids. But such proteins are few in number, apparently, and their characteristic effects may involve some peculiar proportions existing among those amino acids that do occur in them, proportions which cannot be intelligently modified in the absence of reliable data of their exact nature. A lack of such data, in the case of phaseolin, for example, has blocked any attempt to determine the cause of its inadequacy for either maintenance or growth.

Any intelligent supplementing of proteins with isolated amino acids is also impossible in the absence of at least fairly reliable information of their amino acid content. Thus, Osborne and Mendel state[1] that they did not attempt to sup-

[1] Journ. Biol. Chem., 20, No. 3, p. 356, March 1915

plement lactalbumin to determine if normal growth could be secured with less than 9 percent of this protein in the ration, on account, of the inadequate information available as to its amino acid make-up. However, even tho a good knowledge of the amino acid content of proteins were available, due to the evolution of accurate analytical methods, a study of the nutritive value of native proteins and of native proteins supplemented by amino acids, can hardly be expected to reveal the nutritive values of each individual amino acid and especially the quantitative requirements for each individual amino acid. The impossibility of such an attainment by such a method rests in the impossibility of controlling absolutely the amino acid intake and of experimenting with all combinations of amino acids. For example, the interesting and valuable theory of Osborne and Mendel that the maintenance requirements would only involve perhaps one, two, or three, or at any rate a relatively small number of amino acids, is very probably unprovable by their method of experimentation. Thus, while it would be useless to attempt to predict to what

extent scientific ingenuity will overcome the obstacles inherently limiting the applicability of this method to the problems of amino acid requirements, it may be confidently asserted that only a very incomplete solution can be expected from the most extensive experimentation with it.

The only direct method of attacking this problem and the only method with unlimited possibilities, is the method of feeding isolated amino acids, to the complete exclusion of proteins or any of their intermediary degradation products. It is the main purpose of this investigation to test the feasibility of this method of experimentation.

So far as could be ascertained from an exhaustive review of the literature the only attempt on record to cover the nitrogenous requirements of the animal body by substituting the proteins of a ration by a mixture of isolated amino acids has been made by Abderhalden.[1] After many unsuccessful attempts to feed mixtures

[1] Zeitsch. physiol. Chem., 77, pp. 22-58, 1912.

of purified amino acids to dogs, Abderhalden records one experiment of 8 days duration in which encouraging results were obtained. The difficulties encountered in these attempts were, first, inducing the animals to consume the rations containing known amino acid mixtures, and, second, avoiding the digestive disorders which such rations were especially liable to produce, such as vomiting and diarrhoea. By feeding the experimental animal often with small portions of food, partial success was finally secured. In the 8 day experiment above mentioned, the following amino acid mixture was fed: 2 percent of l-cystine, 3 percent of l-serine, 5 percent of glycocoll, d-valine, d-isoleucine, l-aspartic acid, l-phenylalanine, l-tyrosine, l-lysine (as the carbonate), d-arginine (as the carbonate), l-histidine, and l-tryptophane, 10 percent of d-alanine, l-leucine, and l-proline, and 15 percent of d-glutamic acid. Of the amino acids commonly occurring in proteins, this includes all but oxyproline. The day's ration consisted of 25 grams of this amino acid mixture, 50 grams of a glycerine - fatty-acid mix-

ture, 2 grams of cholesterine, 50 grams of glucose, and 5 grams of bone ash, the amino acids constituting about 9 percent. Tho the ration possessed no decided taste, it was a matter of great difficulty to induce the animal to consume it. During the 8 days of feeding, the body weight was constant. A negative nitrogen balance was obtained on the fourth, fifth, sixth, and eighth days. During the subsequent 7 day period, when 25 grams of hydrolyzed beef (with a much smaller percentage of nitrogen) were substituted for the amino acid mixture in the above ration, the body weight increased from 8200 to 8260 grams and a much more favorable nitrogen balance was secured.

Another experimental animal was fed for 6 days with a ration containing 22.1 grams of the above amino acid mixture, 2 grams of hydrolyzed nucleic acid, 25 grams of fat, 20 grams of sucrose, 20 grams of glucose, and 5 grams of bone ash. The weight was practically constant. Negative nitrogen balances were obtained on the third, fourth, and fifth days of feeding. The experiment was terminated by the refusal of the dog to consume any more of the ration. Substitution of the amino acid mixture by hydrolyzed meat in the above ration was followed by favorable results, as in the preceding case.

Abderhalden concludes from these two feeding periods that no physiologically indispensable amino acid is absent from the mixture fed. This conclusion however, must be considerably discounted by reason of the shortness of the periods of feeding and by the unsatisfactory nitrogen balances as compared with feeding periods in which obviously adequate rations were fed, tho no increases in weight were obtained. Thus, in Experiment V, from the first to the forty-seventh day, during which hydrolyzed preparations of complete proteins were fed, the body weight was remarkably constant (only a small increase being recorded during a short period) and the nitrogen balance was consistently positive.

The objections inherent in such short feeding periods as occur thruout Abderhalden's investigations are well illustrated in his Experiment VI. For eleven days (from the thirty-fourth to the forty-fourth inclusive) the body weight of

the animal was practically constant, tho the nitrogen balance was consistently negative (with the exception of that of the fortieth day). Obvicusly such a relation could not exist indefinitely. Such feeding periods as this, and as those in which the amino acid mixture was fed, characterized by an inconsistent relation between body weight and nitrogen balance, must be considered of ambiguous significance.

Further evidence of the unreliability of short feeding experimentsis afforded by the work of Miss Wheeler[1]. Quoting this author:

[1] Journ. Exper. Zoöl. 15, No. 2, pp. 209-223, August 1913.

"Animals have a surprising power of getting along for a time on a qualitatively inadequate diet. Mice kept upon a ration in which two-thirds of the protein was gelatin and the other third casein did not show a decline in body weight for twenty-one days, altho the ultimate decline of every animal fed upon this food showed it to be insufficient; another group made material gains in weight for seventeen days on a diet upon which all ultimately lost weight rapidly and died unless the food was changed."

In one of the papers[2] of Osborne and Mendel, the following significant remark

[2] Journ. Biol. Chem., 13, p. 246, November, 1912.

occurs: "If we had been content to discontinue the experiments after a reasonable period, many of the declines (in body weight) evidently associated with imperfections in the dietary, and readily checked by a change in feeding, would have escaped attention."

II. EXPERIMENTAL INVESTIGATION

1. Selection of Experimental Animals

The arguments in favor of the use of the smaller experimental animals in nutrition work have been well summarized by Osborne and Mendel[3]:

[3] The Carnegie Institution of Washington, Publ. No. 156, Pt. I, p. 6, 1911

"The preparation of suitable pure food supplies on a scale sufficient for long periods and in economically procurable amounts is thereby rendered possible. The necessary scientific measurements and analyses can be conducted on a scale impossible for larger animals, and the problem of care and attention is equally simplified. Experiments can be duplicated without great effort and individual peculiarities eliminated by force of numbers. Furthermore, the various stages of growth and maturity are completed in the smaller animals within relatively short periods of time, so that the permanent effects of any dietary become apparent within a range of days or months that is not outside of ordinary experimental possibilities of observation. As illustrations of some of these features it may be recalled that the ultimate effects of complete inanition are apparent in four or five days in rats or mice; in dogs the fatal outcome may be delayed for many weeks. The duration of life in the white rat is about three years; about 280 days suffice to complete the entire period of growth to maturity."

Since in the experimental project reported below the main limiting factor is the amounts of amino acids that could be bought or prepared, white albino mice were chosen as the experimental animals in preference to rats. Albino mice are only about one-tenth as heavy as rats of corresponding age and therefore require a much smaller amount of food for maintenance and growth. Their span of life is also shorter than that of the rat. It has been stated that the average life of a white mouse is a little less than two years, that they are sexually mature at two months of age and fully grown at 150 days or even less. It would therefore appear that the effect of any inadequacy in a ration would be manifested in an even shorter period of time with mice than with rats.

It is a matter of considerable importance to consider the suitability of white mice as experimental subjects, not only from the standpoint of experimental convenience, but also from the standpoint of the general biological significance of the experimental results obtained. It is of no economic importance or of no human interest simply to determine the qualitative or quantitative food requirements of such animals as rats or mice, if such requirements have no broader application than to those particular species of animals.

It is a generally accepted practice among physiologists and investigators in nutrition and related fields (perhaps too generally accepted) to attach a general biological significance to experimental results obtained upon one particular species, or more truly, upon a few representatives of one particular species

of animals. As a recent illustration of this practice, I will cite the valuable work of Folin and Denis on the distribution and ultimate fate of ingested amino acids in metabolism. The results of these valuable investigations are stated and are accepted as general biological facts, at least as regards mammalian metabolism, with no reference whatever to the fact that they were obtained with one particular species, i.e., cats. The main justification of this practice is that, tho no systematic investigations on the comparative physiology or metabolism of the different species of mammals has been attempted, the experimental findings upon a large variety of animals are all remarkably consistent with one another, if not actually confirmatory of one another. Those differences that have been found in the metabolism of different species of mammals, are largely quantitative and not qualitative. Therefore, it has been concluded, and I think justly so, that experimental results obtained with one species of mammals may be considered as at least qualitatively applicable to mammals in general.

One is apt to have the impression, probably largely on account of the great discrepancy in size between rats and mice and animals of any economic or other importance to man, that these smaller experimental animals are inherently unsuitable for investigational purposes, because any results obtained with them must possess a very limited significance. As a matter of fact, however, it has been shown that rats, and presumably mice, since they are closely related to rats, bear a very intimate relation physiologically to at least one of the larger mammals, i.e., man himself. Thus, Folin and Morris[1] have analyzed the urine of rats, with the following

[1] Journ. Biol. Chem., 14, No. 5, pp. 509-515. June, 1913.

ing interesting results:

".....the percentage composition of rat urine differs but little from that of human urine. Being small animals but voracious feeders the total nitrogen per kilo of body weight is much larger than in the case of man. The percentage relationship between the amounts of total nitrogen, urea nitrogen, and ammonia nitrogen is almost the same as in man. Creatinine nitrogen shows a somewhat larger value for the rats, reaching 15 mgms. per kilo in the mature rat, while man usually eliminates 7-11 mgms. Small amounts of creatine were always found in the urine. This is rather interesting in view of the excessive feeding of rats and

the suggestion of Folin and Denis that the creatine in the urine of children might be due to an 'excessively high level of protein consumption'.

"The most striking and interesting feature of the analyses is the fact that the urine of rats contains quite as much uric acid in proportion to body weight as does human urine. Thru the investigations of Wiechowski and Givens we have learned that mammals other than man convert the greater part of the uric acid into allantoin ..... The purine metabolism of rats is, therefore, like that of man and unlike that of other mammals hitherto investigated."

The work of Donaldson[1] on the comparison of the growth of the albino rat with

[1] Boas Memorial Volume, pp. 5-26.

that of man has shown that "the growth curve of the rat exhibited all the phases found in the human growth curve, and, further, that the curves for the two sexes were similarly related in both the forms examined." From the results of another investigation[2] Donaldson concludes:

[2] Journ. Comp. Neurol. and Psychol., 18, pp. 345-389, 1908

"that man and the rat are similar in the weight relations of their brain and spinal cord, in the form of the growth curves of the brain, in the fraction of the span of life taken for the rapid growth of the brain, and in the proportional development of the brain and cord during this phase. They differ, however, in the intensity of the general growth processes, which are some thirty times more rapid in the rat than in man, in the relation of the completion of the phase of rapid growth to the appearance of puberty and in the longer continuance of the phase of slow growth in the rat."

The close parallelism between the results that Hart and McCollum[3] have ob-

[3] Journ. Biol. Chem., 19, No. 3, pp. 373-395, November 1914

tained with pigs and white rats in their study of the influence of restricted rations on growth also testify to the general biological significance of experimental results secured with the smaller experimental animals. Thus, by supplementing a ration of corn meal and gluten feed, which had proved itself inadequate for the normal growth of pigs, in such a way as to make the total mineral content of the ration approximately like that of a successful salt mixture used in artificial rations with rats, it was found to be adequate for a rate of growth of pigs approximating that of the normal curve for pen-confined animals. Similarly, when the

wheat kernel supplied all the nutrients in the ration, growth was limited with both pigs and rats, and when the wheat kernel was fortified with salts and butter fat, the growth curve was very much improved in both species, altho a normal curve was not secured. However, when the wheat kernel, salt, and butter fat ration was supplemented with casein to the extent of 2.5 percent of the ration, a normal curve of growth was secured with both swine and rats.

From this mass of evidence it would seem extremely probable that the amino acid requirements of rats and mice are closely representative of those of all mammals, certainly qualitatively and possibly to some extent quantitatively.

2. Basal Rations for Maintenance

The excellent results obtained by Osborne and Mendel with their artificial rations was a strong argument towards their adoption with necessary modification in this investigation. Thus, they have been able to keep white rats over 600 days and thru three generations on a ration consisting of the protein, edestin (18 percent), "protein-free milk" (28 percent), starch (24 percent), lard (12 percent), and centrifugalized-butter-fat (18 percent). Miss Wheeler[1] has shown that mice

[1] Journ. Exp. Zoöl., 15, No. 2, p. 211. August 1913.

can be maintained in good health on such a ration for more than six months. According to the results of this investigation, maintenance in apparent good health can be secured with white mice for at least four months on a ration containing 18 percent casein, 28 percent "protein-free milk", 26 percent starch, 10 percent lard and 18 percent purified butter fat. There was no indication at the end of this period that continued maintenance might not be secured for an indefinite period.

The main objection to such a ration in an experiment of this nature is the fact that the "protein-free milk" as prepared according to the directions of Osborne and Mendel always contains nitrogen. These investigators have found percentages of nitrogen ranging from 0.50 to 0.75 in their own preparations. A rather complete analysis of one of their preparations[2] containing 0.70 percent of

[2] Zeitsch. physiol. Chem., 80, p. 316, 1912

nitrogen, gave 0.16 percent of insoluble nitrogen consisting essentially of residues of casein and lactalbumin that were not precipitated, and 0.54 percent of soluble nitrogen. Of the latter 0.18 percent was precipitated by tannin, 0.19 percent on saturation with $ZnSO_4$, and 0.24 percent was precipitable by phosphotungstic acid. They conclude from these results that their "protein-free milk" contains about 2.22 percent of protein of which about 1 percent, or a little less than half, is insoluble in water. In a ration containing 28 percent of this preparation -- the most favorable percentage found by Osborne and Mendel -- about 0.6 percent of the ration would consist of protein from this source.

We have investigated the effect on the nutritive value of "protein-free milk" of a slight modification in its preparation.[1] After precipitating the casein from milk by a slight excess of HCl, filtering off the coagulum, boiling for one-half minute to coagulate the lactalbumin and filtering off this precipitate, the solution was carefully neutralized and again filtered, the filtrate being then evaporated to a thick paste on the water bath at 70° and dried in a current of warm air. The last filtration, which constitutes the modification introduced, presumably would remove the protein insoluble in water, and according to one determination, the percent of total nitrogen was reduced from 0.78 to 0.61 by this procedure. Some of the mineral salts are also removed. Its nutritive value for maintenance, however, is probably not impaired much if at all. In feeding experiments lasting as long as five weeks we have secured as good results with filtered "protein-free milk" as with the unfiltered. Before final conclusions can be deduced, this result, of course, must be confirmed by much longer feeding periods.

[1] All of the "protein-free milk" used in this investigation was prepared from skim milk powder. This product was dissolved in 12 times its weight of water before removing the casein.

In attempting to do away entirely with the " protein-free milk", we have substituted for it in the ration 4 percent of a salt mixture recently used by McCol-

lum[1] on rats with considerable success, and 24 percent of lactose. Most of the

[1] Journ. Biol. Chem., 19, No. 2, p. 250. October 1914. The salt mixture contained the following constituents:

NaCl	0.173 gram
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4 . H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2 . H_2O$	0.540
Calcium lactate + $5H_2O$	1.300
Iron lactate	0.113

mice put on this ration began to show effects of malnutrition and to decline in weight after one or two weeks, some were maintained in apparent good health for three weeks, while, in the most successful experiment on two growing mice of an initial weight of 15.9 grams and 11.5 grams, maintenance and even an increase in weight to 16.8 grams and 19.3 grams respectively were obtained in a feeding period of about 70 days duration, before malnutrition was evident. The results of this test are given in Chart I. The two mice whose body weight curves are shown in this chart were confined in the same cage and the food record applies to both. The prompt recovery of the mice when a ration containing "protein-free milk" that had been filtered after neutralization as described above, was given them on the 83rd day of the experiment is interesting as further proof of the adequacy of this preparation. Taken all in all, the results of the few tests we have made with this salt mixture indicate that it is unsuitable for covering the mineral requirements for the maintenance of white mice for any extended length of time.

Miss Wheeler has shown that Röhmann's[2] salt mixture also when fed with the Osborne and Mendel rations is distinctly inadequate for mice. The remarkably good results obtained by Röhmann himself are inexplicable except on the assump-

[2] Biochem. Zeitsch., 64, pp. 30-62. June 13, 1914.

tion that some of his food materials, which were not by any means carefully purified, commercial protein, for example, being used, were not salt-free. His salt-mixture has been shown by Osborne and Mendel to be also inadequate for white rats.

While the Osborne and Mendel casein and "protein-free milk" rations appear to be ideal for the maintenance of weight and health of white mice, they do not appear to be particularly favorable for the production of offspring. Comparatively few cases of pregnancy were observed with mice on these rations. Where conception occurred, it was generally premature, apparently, and the young were generally eaten by the mother within twenty-four or forty-eight hours. Only three young (out of a litter of five) were successfully raised until weaning on such rations, and in this case the growth during the nursing period was slower than normal. The growth curves of two of these mice subsequent to weaning are given in Chart I.

3. Basal Rations for Growth.

It has been shown by Miss Wheeler that the Osborne and Mendel casein + "protein-free milk" ration does not support normal growth in mice. In fact, practically no growth was secured in most cases. That this difference is due not to qualitative differences in the growth requirements of rats and mice but simply to differences in the proportions of the food constituents in rations suitable for normal growth is indicated by her experiments on milk powder rations.[1]

[1] Journ. Exp. Zoöl., 15, No. 2, pp. 220-221, August 1913

A ration consisting of 60 percent of milk powder, 16.7 percent of starch, and 23.3 percent of lard has been shown by Osborne and Mendel to be sufficient for normal growth in rats. With mice, Miss Wheeler obtained only a slight growth with this ration. However, a ration consisting of 50 percent of milk powder, 7.7 percent of lard, 25.0 percent of "protein-free milk", and 17.3 percent of casein was capable of supporting normal growth with mice. This ration contains the same constituents as the former, with the single exception of starch, but these constituents are present in different proportions. In the latter ration as compared with the former, the protein has been increased by the addition of casein, and the ash and accessory substances by the addition of "protein-free

milk". It is hardly probable that the occurrence of starch in the former but not in the latter ration would indicate a qualitative difference in the growth requirements of the two species. Miss Wheeler was unable to utilize the information obtained by experiments on milk-powder rations in preparing a ration from isolated food substances that would support normal growth of mice. In a recent publication[1] this investigator has secured normal growth of mice on a ration con-

[1] Amer. Journ. of Dis. of Children, 9, pp. 300-317. April 1915

sisting of mixtures of proprietary infant foods, casein, and milk, containing 18-21 percent of protein and 3 to 4 percent of ash. Since the Osborne and Mendel ration for rats contains 18 percent of protein and about 4 to 4.5 percent of ash, it is evident that the unsuitability of this ration for mice does not rest on an insufficient supply of these constituents. Probably it is deficient in the so-called "accessory substances" which are known to be necessary for normal growth. The probability of this conclusion is enhanced by the work of MacArthur and Luckett[2]

[2] Journ. Biol. Chem., 20, No. 2, pp. 161-174. February 1915

who obtained a growth approximating the normal by supplementing the Osborne and Mendel ration with a small amount (5 percent) of a cold alcoholic extract of egg yolk. The finding of these investigators that butter fat does not promote normal growth in mice as in rats may be explained on the assumption that it does not contain a sufficient concentration of the unknown growth-accelerating substances, but that an alcoholic extract of egg yolk is very rich in them. This suggestion is in line with the recent investigation of Osborne and Mendel[3] in which it

[3] Journ. Biol. Chem., 20, No. 3, pp. 379-389. March 1915.

was demonstrated with rats the different natural fats contain different percentages of such growth accelerators.

It has also been demonstrated in this investigation that the Osborne and

Mendel ration, consisting of 18 percent casein, 28 percent "protein-free milk," 26 percent starch, 10 percent lard, and 18 percent butter fat is insufficient for the normal growth of white mice. It is evident from the results obtained that the insufficiency of this ration is the greater, the smaller the mouse. Thus, with very young mice weighing from 8 to 12 grams, practically no growth was secured, while with mice weighing 15 grams or above, a slow growth sometimes resulted.

The first attempt to modify this ration to meet the requirements for the growth of mice consisted in raising the casein from 18 to 34 percent and the "protein-free milk" from 28 to 38 percent. The starch was thus entirely eliminated. This modified ration produced a fairly rapid growth in the mice. The growth curves of this experiment are given in Chart II. The decrease in weight of these mice from the 66th to 68th day was due to a change to another ration which they hardly touched. The nature of this ration is immaterial to the point under discussion. The growth curve indicated in the chart by a broken line is the normal growth curve for mice as determined by Miss Wheeler. According to the latter, the mice grew at a normal rate for about thirty days but failed to attain a normal adult weight.

The second attempt to modify the Osborne and Mendel ration to meet the growth requirements of mice consisted of a substitution of 4 percent of a cold alcoholic extract of dried egg yolk for 4 percent of butter fat, according to the suggestion of MacArthur and Luckett, and, incidentally, a substitution of 0.5 percent of cystine for 0.5 percent of casein. These changes were made in the ration just discussed containing 34 percent of casein and 38 percent of protein-free milk. The curves contained in Chart III illustrate results produced by this second ration. After 14 days on a ration containing 34 percent of casein, 4 percent of the McCollum salt mixture described above, and 4 percent of an alcohol extract of egg yolk, a rapid rate of growth was initiated by a change to the ration under discussion. In this case also, however, a cessation of growth occurred before a normal adult weight was attained. A change on the thirty-eighth day to the ordinary Osborne

and Mendel ration, produced no noticeable change.

In Chart IV are given the growth curves of two mice on a ration of 18 percent protein, 40 percent "protein-free milk", and 4 percent alcohol extract of egg yolk. After an initial decline in weight for 4 to 6 days, a rapid rate of growth resulted which does not appear to have come to a complete standstill at the time this is being written.

The results described above constitute the most successful attempts of this investigation to devise a ration conforming to the growth requirements of mice. They do not apparently constitute a complete solution of this problem. More work is undoubtedly required in this direction.

4. Sources of the Amino Acids used in this Investigation

Most of the amino acids used in this investigation were obtained from hydrolyzed protein material according to the usual methods of preparation. In the following tabulation information concerning the particular methods used is given:

(a) Tyrosine. Prepared by concentration of a neutral solution of hydrolyzed casein or silk. In the case of hydrolysis with HCl, the excess acid was removed by repeated concentration in vacuo, and the last traces practically removed by treatment with lead oxide. In the case of hydrolysis with H_2SO_4, the acid was quantitatively removed by precipitation with $Ba(OH)_2$. The product obtained on concentration of the neutral solutions was dissolved in boiling water, decolorized with animal charcoal, and evaporated to crystallization. The preparation used in this work tested 7.83 percent of nitrogen, the theoretical percent being 7.74.

(b) Cystine. Cystine was prepared from washed wool[1] either by the method of Folin[2], or by the method given by Abderhalden[3]. The latter method gave better

[1] The author's thanks are due to Professor W. C. Coffey for his kindness in supplying an unlimited quantity of wool for this purpose.

[2] Journ. Biol. Chem., 8, pp. 9-10. 1910.

[3] Physiologisches Praktikum, pp. 98-99. Berlin, 1912

yields than the former in our hands. The preparation used in this investigation, which was a mixture of the products obtained by both of the above methods, gave 11.73 percent of nitrogen, the theoretical percent being 11.67.

(c) Glutamic Acid. The hydrochloride was prepared from wheat gluten according to the method of Osborne and Gilbert.[1] The acid was liberated from its hydro-

[1] Amer. Journ. Physiol., 15, pp. 333-334. 1906.

chloride by dissolving in water, making slightly alkaline with aqueous ammonia, and concentrating on the water bath until crystallization begins. Another crop of crystals was obtained from the mother liquors by precipitation with absolute alcohol.[2] The purity of this product was not tested, tho it was very probably

[2] Abderhalden: Physiologisches Praktikum, p. 100. Berlin 1912

a very good preparation.

(d) Arginine, Lysine, and Histidine. These diamino acids were prepared at first according to the method of Kossel and Kutscher as used by Osborne.[3] The

[3] See Plimmer: The Chemical Constitution of the Proteins, Part I, pp. 35-38, 1912

arginine was isolated as the free base instead of the nitrate. The lysine was first isolated as the picrate, and was subsequently liberated from this compound by solution in water, addition of a slight excess of H_2SO_4, extraction of the free picric acid by repeated treatment with ether, removal of the H_2SO_4 in the aqueous solution with $Ba(OH)_2$, and evaporation of the filtrate to dryness. Histidine was isolated as the free base from hydrolyzed blood meal according to the usual procedure. Later some of the modifications of Van Slyke[4] were followed, notably the

[4] Journ. Biol. Chem., 16, No. 4, pp. 533-535. January 1914

detailed procedure for the phosphotungstic acid precipitation, the decomposition of the diamino acid phosphotungstates and later the decomposition of the lysine

phosphotungstate by solution in ammonia and treatment with $Ba(OH)_2$. The lysine and histidine preparations were not tested. The arginine preparation gave 30.50 percent of nitrogen, instead of 32.18, probably due to a contamination with histidine, or possibly to slight decomposition on evaporation to dryness. It was considered good enough for the preliminary work of this investigation.

(e) Tryptophane. This amino acid was prepared according to the method of Hopkins and Cole.[1] All of the preparations used in this investigation were not

[1] Journ. of Physiol., 27, pp. 420-421. 1902

tested. The only one tested gave 13.68 percent of nitrogen, the theoretical being 13.46.

(f) Proline. The proline used in the feeding experiments described below was prepared from a desiccated preparation of hydrolyzed casein by extraction with absolute alcohol, evaporation of the extract to dryness, extraction of the residue with absolute alcohol, and further repeated evaporation and extraction until no residue remained upon treating with alcohol. The purity of the product obtained in this way was not tested.

The amino acids listed below were synthesized:

(g) Valine. dl-Valine was prepared by the following steps: (1) Oxidation of isobutyl alcohol to the aldehyde by $K_2Cr_2O_7$ and H_2SO_4,[2] (2) condensation of the

[2] Monatschr., 2, p. 614, ibid. 4, p. 660.

aldehyde with KCN and NH_4Cl[3] in a bomb at a temperature of 50^0 to 60^0, and (3)

[3] Berichte, 39, p. 1726

saponification of the cyanhydrin by HCl. The preparation used was not tested for purity. The yields were fairly good.

(h) Serine. For the preparation of dl-serine, chloracetal served as the starting point. The method of Leuchs and Geiger[4] was followed thru the prepara-

[4] Berichte, 39, pp. 2644-2699. 1906

tion of ethoxylacetaldehyde. The latter compound was condensed in a bomb with NH_4Cl and KCN at 50° to 60°. The saponification of the cyanhydrin, the removal of the ethoxyl group, and the isolation of the final product were performed according to the original method. For reasons at present unknown, the only synthesis attempted by this method gave an almost infinitessimal yield. The method is being investigated further.

The remaining amino acids used in this investigation were Kahlbaum's products.[1]

[1] I take pleasure in acknowledging the kindness of Mr. C. G. MacArthur of the Chemistry Department in furnishing us with many of these amino acids from his stock on hand, Without his timely assistance the scope of this investigation would necessarily have been curtailed.

5. Other Experimental Details

The mice were confined in galvanized iron cages, 8 in. in diameter and 6 in. high. In most of the experiments two mice were confined in a single cage. A small amount of paper excelsior was always kept in the cages, and with the exception noted below the cages stood in shallow tin pans for the collection and removal of the excreta.

The rations were placed in small porcelain crucibles 1 3-4 in. in diameter at the top and about 1 1-8 in. in height. To prevent undue scattering of the food, the top of the crucible was fitted with a flanged ring of tinned copper, the hole in the ring being about 1 in. in diameter. This was crossed by two wires at right angles to each other, so that there were four holes thru which the mice could insert their heads but not much else. With this device it was hoped that all scattering of the rations would be avoided, but almost invariably with the amino acid rations considerable scattering would occur in spite of all precautions. In such cases to insure a fairly accurate determination of the food intake the cage was placed over a crystallizing dish which fitted under it tightly. This prevented the inroads of any wild mice. To insure a fairly good separation of food waste and urine, a piece of heavy filter paper about 4 in. square was placed immediately

under the food crucible, in the crystallizing dish. With these arrangements it was rarely that a good determination of the food intake could not be made though only at the expense of much time and patience. The food was weighed out once a day, at which time the uneaten food from the preceding day was determined.

Water was supplied the mice from an inverted bottle attached to the top of the cage, provided with a bent delivery tube protruding into the cage. This method is very satisfactory, if care is taken to insure that the delivery tubes are always full of water. Distilled water only was used.

The mice were kept in light and dry quarters. During the autumn months, when no artificial heat was available, the temperature occasionally fell to a few degrees below 70° Fahrenheit, but later after the installation of a radiator, the temperature always remained above 70°, and was generally maintained between 75° to 80°. The cages were cleaned out once a day in most cases and at least once every other day in all cases. They were sterilized whenever such measures were required, by passage through a hot solution of strong alkali and thorough rinsing with water.

It was necessary for the proper interpretation of the results secured with the amino acid rations to determine the minimum energy requirements for maintenance. This was done by the use of the ordinary Osborne and Mendel ration containing 18 percent of casein and 28 percent of fat. Mice were fed restricted amounts of this food starting with 4 grams per day for two mice and gradually lowering the intake until the body weight was affected. These experiments are still in progress, and while a definite minimum cannot be deduced from the results at present, it appears probable that it very closely approximates to 8 grams of ration per day per 100 grams of body weight. Since all the experimental rations possess approximately the same energy value, this figure may be tentatively applied in the case of each.

6. Feeding Experiments with Amino Acids.

In view of the fact that growth probably sets a much higher standard as regards amino acid requirements than does simple maintenance, the logical point of

attack in this investigation appeared to be the amino acid requirements for maintenance. Another argument in favor of this procedure was that adult mice, which would be used in maintenance work, could probably withstand the digestive disturbances anticipated in the feeding of amino acid rations, better than immature animals.

In the first attempts to cover the nitrogenous requirements of mice by definite mixtures of amino acids, the Osborne and Mendel ration was modified simply by the substitution of the 18 percent casein by 18 percent amino acids. The amino acid mixtures used are given in the appendix. The proportions of the individual amino acids included in these mixtures were more or less arbitrarily chosen, since there is little data at hand that would logically furnish a presumption in favor of any one mixture as opposed to others. In preparing these mixtures some consideration was given to the analyses available of pure proteins known to be complete for maintenance and growth; some consideration was necessarily paid to the stock of amino acids on hand.

Before considering the experiments with isolated amino acids, attention is first directed to an experiment on the feeding of hydrolyzed casein, the results of which are given as Chart V. All preparations used were obtained by the action of pancreatin on casein in 0.8 percent Na_2CO_3, the proportions of the materials being 100 grams of pancreatin to 1 kg. of casein in 10 liters of carbonate solution. This mixture was preserved with toluol during incubation. After several weeks hydrolysis at 35° to 38°, the solution was filtered water-clear, evaporated to a sticky consistency on the water bath at a low temperature (70° or less), and dried to a brittle mass in a current of warm air. The material prepared in this way could be pulverized easily and was passed through a 40 mesh sieve before using.

Two female mice were put on a ration containing 18 percent of this casein digest, 3.7 percent of McCollum's salt mixture, 20 percent starch, 30.3 percent lactose, 18 percent butterfat, and 10 percent lard. On this ration the heavier mouse maintained itself for 31 days. The other mouse at about the twelfth day began to show signs of malnutrition, such as a rough coat, emaciation, and rest-

lessness, and declined gradually but steadily in weight to the 31st day. At this point the ration was changed to one containing 18 percent of the casein digest, 28 percent "protein-free milk", 26 percent starch, 18 percent butterfat, and 10 percent lard. The first mouse continued to maintain its weight on this ration. The second mouse immediately and rapidly increased in weight and lost all symptoms of malnutrition. The favorable effect of the substitution of "protein-free milk" for lactose + the McCollum salt mixture is clearly evident with this mouse.

The casein digest used in these first two rations gave a very slight biuret reaction, and a precipitate in aqueous solution with tannic acid, HNO_3 after saturation with NaCl, $K_4Fe(CN)_6$ and acetic acid, and picric acid, indicating the presence of at least traces of proteoses and peptones. Even on long continued incubation with frequent additions of small portions of pancreatin, we were unable to obtain a solution which after filtration, gave negative tests with the above reagents. In order to remove the proteoses and peptones, the filtered solution (after several weeks incubation) was precipitated with 9 1-2 volumes of 95 percent alcohol, filtered, and dried as explained above. The product thus obtained gave negative results with the above reagents, even after standing in contact with them for 15 or 20 hours.

On the 48th day of the experiment the ration was changed to one containing 12 percent of this completely hydrolyzed product, 6 percent of sucrose, 28 percent of "protein-free milk", 26 percent of starch, 18 percent of butterfat, and 10 percent of lard. For the following 8 days the mice decreased gradually but steadily in weight. Subsequently one mouse recovered its original weight, while the other maintained its weight at the lower level. On the 74th day of the experiment the casein digest in the ration was decreased from 12 to 10 percent and the sucrose increased from 6 to 8 percent. At the present writing these mice are apparently in perfect nutritive dondition, after a period of 78 days of observation. During the first 48 days their nitrogenous requirements were covered largely by an unknown mixture of amino acids, while during the last 30 days, the nitrogenous requirements

were covered presumably entirely by amino acids or simple derivatives of amino acids. This statement does not take into consideration the nitrogenous compounds of the "protein-free milk", part of which are known to be protein in nature.

The results of the feeding experiments with rations containing 15 to 18 percent of mixtures of isolated amino acids are shown graphically in Charts VI to XV inclusive. It should be stated at the outset that the determinations of the food intake in these experiments are questionable. In these tests the cages were placed over tin pans which were open to the inroads of wild mice known to infest the room in which the work was carried on. The food was invariably scattered into the pans. Losses due to the possible consumption of this food by the wild mice and to the mixture of urine with it were thus unavoidable. Only the best determinations of food intake are therefore given, and even these must be considered as maximal in all cases. The daily feed record curve is therefore very incomplete in many cases.

All of these amino acid rations in the quantities consumed were inadequate for maintenance. In some cases, notably in Charts VI and IX, only a slow decline in weight accompanied the consumption of these rations; in other cases, as in Charts VIII and XII, a rapid decline resulted. In several cases the mice died inside of two weeks (see Charts X, XIII, and XV), and in other cases after longer periods.

In Charts VI and IX the mice were kept alive for 29 and 27 days respectively on amino acid rations, losing in this time, 30 to 40 percent of their original weight. The condition of these four mice at the end of 29 and 27 days was obviously poor. Their hair was rough, emaciation was extreme, and they were weak and barely able to use their hind legs. Upon changing to a ration containing 18 percent casein, recovery of the original weight occurred in three cases. In one case recovery was not complete, though the health of the animal was apparently restored.

Four of the mice that died showed symptoms of severe diarrhoea. The other six mice that died on the amino acid rations showed no outward symptoms of digestive disturbance. No post mortem examinations were made of these mice.

The six mice whose weight curves are shown in Charts VI, VIII, and IX, were placed inadvertently upon 18 percent casein rations on the 33rd, 19th, and 27th experimental day, respectively, which contained, instead of the ordinary "protein-free milk" preparation, one that had been thoroughly extracted with a mixture of benzene and absolute alcohol, containing 60 percent of the former to 40 percent of the latter. While on this ration the mice gained only very slowly in weight, in one case only barely maintaining constant weight. One of the mice in Cage 4 (Chart VIII) gained rather rapidly at first and as rapidly declined subsequently. No decided improvement in the general appearance or behavior of the animals was observed and the food intake, in so far as this could be determined, showed no permanent increase. This ration was changed to one containing the same constituents except for the substitution of the ordinary "protein-free milk" for the extracted preparation. The change was made on the 40th experimental day in Cage 19 (Chart VI), on the 26th experimental day in Cage 4 (Chart VIII), and on the 36th experimental day in Cage 7 (Chart IV). In the first case an improvement in the food consumption and in body weight was at once evident. In the second case, these improvements were both very marked, and certainly striking. In the third case an improvement in food consumption was clearly marked, and a slight improvement in the body weight curve is noticeable. In all cases an improvement in appearance and behavior was observable. These results are interesting as indications of increased requirements of the so-called accessory substances for complete recuperation after long periods of malnutrition.

The explanation of the inability of any of these amino acid rations to prevent loss of weight may be either a qualitative inadequacy or simply a consumption of them below the minimum necessary for this purpose. It was possible that the food consumption was so small as to be inadequate to satisfy the energy requirements. In view of the inaccuracy of the determinations of food intake the latter possibility could not be tested except by further investigation. It was obvious, however, that in all cases the mice ate less of the amino acid rations than of the

casein rations.

At this stage of the investigation the publications of Osborne and Mendel and of McCollum and Davis on minimum protein requirements appeared, and a plan for further work with the amino acid rations immediately suggested itself. By lowering the percentage of amino acids in the ration it was hoped that the food intake of the mice could be increased to normal, provided that the diminished consumption of the 15 to 18 percent rations was due simply to some disagreeable flavor imparted to the rations by the amino acids contained in them.

This plan was first tested with a ration containing 8.1 percent of amino acids (Mixture V) and the results are given in Chart XVI. The food intake on this ration was apparently good. The body weight decreased steadily, however, probably due to the fact that only six amino acids were included in the ration. On the ninth day of the test, and through the fiftieth day, non-nitrogenous rations were fed on some of the days and the amino acid rations on the other days. The latter days are indicated on the daily feed record curve by circled points. On some of the days not so indicated mixtures of non-nitrogenous and amino-acid rations were given. From the ninth thru the sixteenth days the non-nitrogenous ration used contained[1] the

[1] This ration contained:

sucrose	18	percent
lactose	24	"
salts	4	"
lard	10	"
butterfat	18	"
starch	26	"

McCollum salt mixture; from the seventeenth thru the fiftieth day a ration containing[2] "protein-free milk" was used.

[2] This ration contained:

sucrose	18	percent
"protein-fr. milk"	28	"
starch	26	"
lard	10	"
butterfat	18	"

The favorable influence of the introduction of the non-nitrogenous rations is

evident. The body weight curve shows only a very gradual decline. The food consumption was much better on the non-nitrogenous than on the amino acid rations, and almost invariably a day's feeding of the former when immediately preceded by a day's feeding of the latter ration produced a more or less clearly marked increase in body weight. For 50 days this mouse was kept alive without the feeding of appreciable amounts of protein, the only source of protein in the rations being the "protein-free milk".[1] Towards the last of the test the animal was in very poor condition.

[1] It might be said at this point that Amino Acid Mixture V (see appendix, for a description of these mixtures) was put into a ration containing the McCollum salt mixture and no "protein-free milk". All subsequent amino acid mixtures, and all previous amino acid mixtures for that matter, were made up into rations containing 28 percent of "protein-free milk". The ordinary procedure was to include a given percentage of amino acids and a percentage of sucrose equal to 18 minus the former percent, the 18 percent of casein in the Osborne and Mendel ration being substituted in this manner.

An attempt to recuperate it on rations containing small percentages of casein supplemented with cystine and tryptophane failed, and the mouse died on the fifty-eighth day of the experiment.

In Chart XVII are shown the body weight curves of a male and female mouse of adult weight that were first placed upon a non-nitrogenous ration containing no "protein-free milk". The weight of these mice declined steadily and rapidly. On the eighth day of the experiment they were put upon a ration containing 8.1 percent of Amino Acid Mixture V, and for the next 23 days this ration was given them alternately with a non-nitrogenous ration, the alternate periods being irregular in length as indicated in the chart. The non-nitrogenous ration first given the mice was used in this way thru the twelfth day of the experiment after which a ration containing "protein-free milk" was substituted for it.

The rapid decline in body weight during the first 10 days of the experiment was distinctly moderated, apparently by the introduction of the amino acid rations, the rate of decline from the tenth to the fortieth day being very gradual. Toward the last of this period two other amino acid mixtures were tested with no observable effects on the body weight curve. On the fortieth day a ration containing

1.88 percent of casein, .06 percent of cystine and .06 percent of tryptophane was given the mice. During 15 days on this ration the weight declined slowly, but steadily, with no improvement in food intake. On the fifty-sixth day a ration containing 18 percent casein was fed. This occasioned an increase in body weight and food consumption. The former increase is still continuing. This latter ration contained a preparation of "protein-free milk" which had been filtered after neutralization of its solution.

The two experiments, the results of which are graphically shown in Charts XVI and XVII, showed clearly that the body weight curves of mice on amino acid rations could be favorably affected by interposing non-nitrogenous rations alternately with the test rations. This favorable effect may be due simply to the fact that the former rations were consumed in larger quantities than the latter so that the energy requirements could be covered, or at least more nearly covered than on amino acid rations entirely. While this regime at the same time lowers the intake of amino acids, it might still be consistent with nitrogenous equilibrium in view of the information recently communicated by Osborne and Mendel and by McCollum and Davis on the remarkably low protein requirements for the maintenance of rats, and presumably also of mice. Therefore, a series of feeding experiments were planned on various amino acid mixtures which were included in percentages ranging from 2.5 to 9 in rations containing 28 percent "protein-free milk", 26 percent starch, 10 percent lard, 18 percent butterfat, and a varying percentage of sucrose, depending upon the percentage of amino acids, as explained above. These amino acid rations were fed alternately with non-nitrogenous rations containing "protein-free milk", in the manner illustrated in the two experiments just described. The results of these experiments are given in Charts XVIII to XXVIII inclusive.

As with the experiments illustrated in Charts XVI and XVII, this series was much more successful in every way than the first series in which 15 to 18 percent amino acids were fed continuously. Altho decreases in body weight resulted in every case in both series of experiments, they were much more gradual in the form-

er than in the latter. Furthermore, signs of emaciation and malnutrition put in an appearance after a much longer period of feeding in the case of those experiments in which the amino acids were fed in small concentration and were not fed continuously. Thus, the mouse whose body weight curve is shown in Chart XIX, after 42 days of amino acid feeding, resulting in a loss of 30 percent of the original body weight, can hardly be distinguished in outward appearance or behavior from an adequately nourished animal of equal size. The same may be said of the mouse whose data are illustrated in Chart XXIV, after 24 days of amino acid feeding, with a resulting loss of about 25 percent of the original body weight.

The mortality on this dietary regime was 1 mouse out of 18 under observation. In the case of that one mouse, the ration contained only 2.5 percent of amino acids, the smallest percentage used, and the amino acid mixture contained only eight amino acids. With the 15 to 18 percent amino acid rations fed continuously, the mortality was 10 out of 20 mice under experiment. This difference is the more striking when it is remembered that the feeding periods with amino acid rations were in general much shorter with the 15 to 18 percent rations than with the rations of lower nitrogen content. Furthermore, no cases of diarrhoea or any other symptoms of digestive disorder were noted, in those experiments involving the feeding of rations of low amino acid content. With the first rations tested this statement certainly does not hold.

Inspection of the eleven charts under discussion does not reveal any clearly marked differences in the general character of the variations in body weight. Some of the curves are seen to be slightly steeper than others, but these differences, according to our current ideas of amino acid requirements, would be considered incommensurate with the differences in the rations fed. In these rations the amino acid content varied from 2.5 to 9 percent, and the amino acid mixtures contained from 8 to 16 individual acids. Most of the mixtures contained both of the amino acids known to be biologically indispensable, i. e., tyrosine and tryptophane. Others, such as Amino Acid Mixtures X, XI, and XII (see appendix) were lacking in

one of these two acids, the first being totally deficient in tryptophane, the second in tyrosine, and the third in both tyrosine and the closely related amino acid, phenylalanine. It was a matter of great surprise that two of the most successful feeding experiments involved the use of these obviously inadequate amino acid mixtures (see Charts XXIV and XXVI)

This general similarity in body weight variations is the more surprising in view of the experiments on the feeding of isolated proteins. Such studies have shown that proteins inadequate for maintenance, due to inadequacies in their amino acid make-up, presumably, exhibit more or less characteristic effects on the body weight. For example, phaseolin leads to a slow decline in body weight, zein to a more rapid decline, and gelatin to a still more rapid decline. Thus, as regards the last two proteins, Miss Wheeler has shown[1] that with zein one-third of the body

[1] Journ. Exper. Zool., 15, p. 215. August 1913.

weight was lost in about 25 days, while with gelatin one-third was lost in 12 days. It is true that the absence of data of the food intake precludes an analysis of the exact significance of such data. However, it is natural to expect that amino acid mixtures characterized by qualitatively different types of deficiencies should exhibit distinct characteristic effects. That such effects were not produced in the experiments reported here is therefore of considerable interest.

In speculating on the causes of the decreases in body weight ultimately obtained with all the amino acid mixtures, the most plausible theory is that in no case was enough of the amino acid mixture consumed to really constitute a fair test of its adequacy. Although the favorable effects of feeding amino acids, not continuously, but alternately with non-nitrogenous rations, is perfectly evident from what has been said above, it seems to be a fact that this alternation of the two types of ration, results in an approximately normal consumption of the non-nitrogenous ration, but in a very small consumption of the amino acid ration, in even a smaller consumption than would apparently result if the amino acid ration were

fed continuously. Thus, while this alternation of rations in most cases assured a sufficient calorie intake, it operated against the attainment of a sufficient amino acid intake, unless this is much smaller than has hitherto been supposed.

It is surprising how the experimental animals could differentiate between rations containing no added amino acids and those containing very small concentrations of amino acids. In Chart XX is illustrated a case in point. The amino acid ration used in this experiment contained only 2.5 percent of a mixture of amino acids. However, as the daily feed record shows, the consumption of this ration was almost invariably lower than that of the non-amino acid ration. It seems impossible that this distinction could have been made on the basis of a difference in taste, though it is difficult to see upon what other basis it could be accomplished.

In an attempt to investigate the relative efficiency of the different mixtures of amino acids in a quantitate way, the data given in Table I were computed, covering the first two weeks of each feeding period. The initial weights of the mice given in Column 3 are averages in each case of the weight on the day immediately preceding the beginning of the experiment and of the weight recorded on the first day. The final weights given in Column 5 are in each case averages of the weights on the thirteenth, fourteenth, and fifteenth days of the feeding periods. It was hoped in this way to give the losses in weight (Columns 8 and 10) more significance than if they had been calculated merely as differences between single weights. The average weights in Column 7 involve all of the daily weights of the two week periods as well as the weight before the experiment started and the weight of the fifteenth day. These average weights were used in computing the daily intake per 100 grams body weight of total food and of amino acids, given in Column 14 and 17.

The relative efficiency of the various amino acid mixtures indicated in Column 2 can be best studied by comparing the percentage losses in weight (Columns 9 and 10) with the daily food intake and daily amino acid intake per 100 grams body weight (Columns 14 and 17). In making comparisons it must of course be realized that the number of animals on any one amino acid mixture was

too small to afford an even approximate idea of the extent of experimental error due to individual variation under the same conditions of feeding. The following discussion must be read with this point continually in mind.

The percentage losses in weight ranged from 12.8 to 32.6. These differences may be in part accounted for by differences in the total intake of food per day per 100 grams body weight. As has been said above, a preliminary and wholly tentative determination of the minimum intake of rations of this general description for maintenance of body weight, has shown that this value is probably close to 8 grams per day per 100 grams body weight. The high percentage losses in weight, such as those for Mice 33♂, 33♀, 44♂, and 49♂, seem therefore to be dependent upon a consumption of food below the minimum needed to cover the energy requirements for maintenance.

The relatively small loss in weight of Mouse 50♂ on an amino acid mixture composed of only 8 acids, was accompanied by a liberal food intake, and compares very favorably with the losses in weight of the mice in cages 32 or 30, on amino acid mixtures containing 11 and 16 acids respectively, though the mice in these latter cages were also apparently ingesting sufficient food to cover their energy requirements for maintenance. The amino acid mixture fed to the mice in Cages 30 and 33 was the most complete in its assortment of acids of any mixture used. The results obtained were among the least favorable of any. The relatively small loss in weight of Mouse 49♂, on a mixture of 10 amino acids not including tryptophane and on an apparently inadequate food intake, as well as the loss in weight of Mouse 48♂, on a mixture of 10 amino acids not including tyrosine, are remarkable and totally unexpected.

A similar comparison to that given in Table 1 is afforded by Table 2 on the basis of the first three weeks of those experiments of such a length or longer. Here also Cages 30 and 33, receiving the most complete mixture of amino acids, show to a disadvantage. However, the differences in weight variation are not so marked at the end of three weeks as at the end of two weeks. The mice of Cage 32 show up to better advantage in Table 2 than any of the others.

TABLE 1. RELATIVE EFFICIENCY OF AMINO ACID MIXTURES. COMPARISON OF FIRST TWO WEEKS OF FEEDING PERIODS.

Number of mouse	Amino acid mixture	Body weight of mice: Initial per mouse	Initial per cage	Final per mouse	Final per cage	Average	Loss in body weight: In grams per mouse	In grams per cage	In percent per mouse	In percent per cage	Weight of food eaten per cage: Total	Average per day	Average per day per 100 g. body weight	Weight of amino acids eaten per cage: Total	Average per day	Average per day per 100 g. body weight
32 ♂	VI	21.0		16.0		18.5	5.0		23.8							
32 ♀	VI	19.8	40.8	16.2	32.2	18.1	3.6	8.6	18.2	21.1	52.6	3.76	10.27	.992	.0707	.193
50 ♂	VII	21.9	21.9	19.1	19.1	20.2	2.8	2.8	12.8	12.8	27.8	1.99	9.85	.327	.0234	.116
34 ♂	VII	19.7		16.1		17.6	3.6		18.2							
34 ♀	VII	19.6	39.3	14.3	30.4	16.5	5.3	8.9	27.0	22.9	36.9	2.64	7.74	.415	.0296	.087
46 ♀	VIII	17.7		14.6		15.9	3.1		17.5							
46 ♀	VIII	17.1	34.8	12.7	27.3	14.8	4.4	7.5	25.7	21.6	40.7	2.91	9.48	.805	.0575	.187
33 ♂	IX	23.8		18.0		20.4	5.8		24.4							
33 ♀	IX	21.1	44.9	16.2	34.2	18.0	4.9	10.7	23.2	23.8	37.8	2.70	7.03	1.032	.0737	.192
30 ♀	IX	21.4		17.3		19.1	4.1		19.2							
30 ♀	IX	23.7	45.1	16.9	34.2	20.3	6.8	10.9	28.7	24.1	48.3	3.45	8.76	1.266	.0904	.229
49 ♂	X	21.2	21.2	18.1	18.1	19.2	3.1	3.1	14.6	14.6	17.8	1.27	6.61	.360	.0257	.134
16 ♂	X	19.9		15.6		17.5	4.3		21.6							
16 ♀	X	23.0	42.9	16.8	32.4	19.6	6.2	10.5	27.0	24.5	43.3	3.09	8.32	.724	.0517	.139
48 ♂	XI	21.5	21.5	18.3	18.3	20.0	3.2	3.2	14.9	14.9	34.8	2.49	12.45	.524	.0374	.187
44 ♂	XII	23.9		16.1		19.5	7.8		32.6							
44 ♂	XII	20.3	44.2	16.9	33.0	18.5	3.4	11.2	16.7	25.3	32.6	2.33	6.13	.756	.0540	.142
49 ♂	XIII	20.2	20.2	15.4	15.4	17.5	4.8	4.8	23.8	23.8	15.0	1.07	6.11	.585	.0418	.239

TABLE 2. RELATIVE EFFICIENCY OF AMINO ACID MIXTURES. COMPARISON OF FIRST THREE WEEKS OF FEEDING PERIODS

Number of mouse	Amino acid mixture	Body weight of mice: Initial per mouse	Initial per cage	Final per mouse	Final per cage	Average	Loss in body weight: In grams per mouse	In grams per cage	In percent per mouse	In percent per cage	Weight of food eaten per day: Total	Average per day	Average per day per 100 g. body weight	Weight of amino acids eaten per cage: Total	Average per day	Average per day per 100 g. body weight
32 ♂	VI	21.0		16.3		17.8	4.7		22.4							
32 ♀	VI	19.8	40.8	16.7	33.0	17.7	3.1	7.8	15.7	19.1	76.9	3.66	10.31	1.372	.0653	.184
46 ♀	VIII	17.7		11.4		13.8	6.3		35.6							
46 ♀	VIII	17.1	34.8	12.8	24.2	15.1	4.3	10.6	25.1	30.5	56.4	2.69	9.31	.945	.0450	.156
33 ♂	IX	23.8		16.0		19.3	7.8		32.8							
33 ♀	IX	21.1	44.9	14.2	30.2	17.0	6.9	14.7	32.7	32.7	55.6	2.65	7.30	1.302	.0620	.171
30 ♀	IX	21.4		16.1		18.2	5.3		24.8							
30 ♀	IX	23.7	45.1	15.0	31.1	18.8	8.7	14.0	36.8	31.0	71.0	3.38	9.01	1.560	.0750	.203
49 ♂	X	21.2	21.2	15.9	15.9	18.4	5.3	5.3	25.0	25.0	29.6	1.41	7.66	.572	.0272	.147
16 ♂	X	19.9		14.9		16.7	5.0		25.1							
16 ♀	X	23.0	42.9	15.3	30.2	18.4	7.7	12.7	33.5	29.6	63.8	3.04	8.66	.996	.0474	.135
48 ♂	XI	21.5	21.5	15.7	15.7	18.9	5.8	5.8	27.0	27.0	47.1	2.24	11.85	.668	.0318	.168
44 ♂	XII	23.9		14.5		18.1	9.4		39.3							
44 ♂	XII	20.3	44.2	16.0	30.5	17.8	4.3	13.7	21.2	31.0	50.3	2.40	6.68	1.164	.0554	.154

A comparison of Mice 32♂, 32♀, and 50♂ on Amino Acid Mixture VI, with Mice 33♂ and 33♀ on Amino Acid Mixture IX for the third and fourth weeks of the feeding periods gives the following data:

	Body Weights			Loss in Weight	
	Initial	Final	Average	Grams	Percent
32♂	16.1	14.7	15.7	1.4	8.7
32♀	16.8	15.3	16.3	1.5	8.9
50♂	19.6	17.3	18.2	2.3	11.7
33♂	17.6	15.3	16.4	2.3	13.1
33♀	15.9	13.5	14.6	2.4	15.1

During these two weeks Mice 32♂ and 32♀ consumed on an average per day per 100 grams body weight 9.94 grams of total food and 0.128 grams of amino acids; Mouse 50 consumed on the same basis 8.52 grams of total food and 0.132 grams of amino acids; and Mice 33♂ and 33♀ consumed 7.46 grams of total food and 0.130 grams of amino acids. The percentage decreases in body weight increase with decreasing consumption of food per day per 100 grams body weight. Whether the character of the amino acid mixtures was also involved cannot be told with certainty.

In order to be in a position to appreciate fully the above experiments, feeding tests were made on three mice, using the non-nitrogenous rations only, containing "protein-free milk". The results of these tests are given in Charts XXIX and XXX. Experiments were also run on two mice with rations containing small percentages of casein supplemented by cystine and later by cystine and tryptophane, and containing the ordinary 28 percent of "protein-free milk". The results are given in Chart XXXI.

When the non-nitrogenous "protein-free milk" rations were fed to three mice continuously, one died at the end of 12 days, one decreased in weight continuously (and especially rapidly after the twenty-second day) for 32 days. On the thirty-second day this mouse showed extreme weakness and emaciation, and severe diarrhoea so that it will be remarkable if it can survive for more than a day or two. The third mouse is exhibiting a slow decline in weight and, after sixteen days of feeding, shows evident signs of malnutrition. On comparing these results with those

above discussed on the amino acid rations it may be said with a good deal of confidence that the alternate feeding of amino acid rations and non-amino acid rations gave better results in regard to body weight variations than the continuous feeding of the non-amino acid rations gave alone if the results obtained with Mice 43♂ and 43♀ are fairly representative of the effect of the latter type of feeding. The body weight curve of Mouse 48♀ will probably follow a similar course to that of the others. This statement receives added significance in view of the greater food intake on the non-nitrogenous ration as compared with the amino acid rations.

Certainly as regards the outward appearances of malnutrition, the alternate feeding of amino acid rations and non-amino acid rations gave more favorable results than the continuous feeding of the latter alone. In the latter case the mice showed a roughening of the hair in a very short time, while in the former case, such an appearance could be deferred for three or four weeks.

The body weight curves of the mice fed rations containing small percentages of added casein, cystine, and tryptophane are quite comparable with those of mice on amino acid rations fed alternately with non-amino acid rations. However, the death of one of the mice after 30 days of feeding taken in conjunction with the liberal food intake of the mice on thecasein rations, would tend to place the advantage again with the amino acid rations when fed alternately with non-amino acid rations.

The results obtained with Mice 30♀ and 30♀ shown in Chart XXIII, are interresting in throwing considerable light on the nutritive value of the "protein-free milk" prepared according to the modification mentioned on page 20 . Previous to the feeding of a ration containing 6 percent of Amino Acid Mixture IX, these mice had been maintained in excellent condition for 38 days on a casein ration containing 28 percent of the modified "protein-free milk". This was followed by 26 days

of feeding on a ration containing 6 percent of Amino Acid Mixture IX[1] + 28 per-

[1] It might be said at this point that Amino Acid Mixtures IX, X, XI, XII, and XIII were all made into rations containing 28 percent of the modified "protein-free milk", with a nitrogen content of.6 percent. Also the non-amino acid rations alternated with these amino acid rations contained 28 percent of the same preparation.

cent of the modified "protein-free milk", fed alternately with a non-amino acid ration also containing 28 percent of this modified preparation. On this regime the body weight decreased continuously. On the twenty-sixth day of the experiment the mice were changed to a ration containing 18 percent casein + 28 percent of the modified "protein-free milk". The weights of the mice immediately and rapidly increased, accompanied by an improved consumption of food. This increase is still continuing and the mice are healthy in appearance and their behavior is entirely normal. It may be said, therefore, that the modified "protein-free milk" has successfully covered the mineral requirements of these mice for 68 days.

III. CONCLUSIONS

1. Summary

The main results of the above investigation may be summarized briefly as follows:

1. The Osborne and Mendel ration containing 18 percent casein, 28 percent "protein-free milk", 26 percent starch, 10 percent lard, and 18 percent purified butterfat, is suitable for the maintenance of mice for an indefinite period in good health. However, it does not appear to be as suitable for mice as for rats, since successful reproduction and nursing of the young seems to be only exceptionally accomplished when adult mice are being fed this ration.

2. The main objection to the use of Osborne and Mendel's "protein-free milk", i.e., the presence of protein material in it, has been in part obviated by a modification in its preparation. This modification consists in filtering the solution from the precipitate produced on neutralization, after the removal of the

casein and albumin. This filtration apparently removes the protein residues insoluble in water, which Osborne and Mendel have shown constitute about one-half of the protein content of "protein-free milk", and reduces the nitrogen content 0.10 to 0.20 percent. Our experience in feeding this modified preparation indicates that it has not lost any of its efficiency in covering the mineral requirements for maintenance or in supplying certain necessary accessory substances.

3. The above mentioned ration of Osborne and Mendel is for some reason unsuitable for the production of normal growth in mice. Our most successful modification of it for this purpose consisted in increasing the "protein-free milk" from 28 to 40 percent, and in substituting 4 percent of the butterfat by 4 percent of a cold alcohol extract of egg yolk. The growth obtained with this ration approximates closely to the normal.

4. Two mice have been maintained in good condition for 48 days on a preparation of enzyme digested casein containing some proteoses and peptones, and for 30 days on a ration containing 10 to 12 percent of a casein digest from which the proteoses and peptones had been completely removed by precipitation with alcohol. This feeding is still continuing and the mice are in excellent condition and appear to have a perfectly normal appetite.

5. Experiments on the feeding of 15 to 18 percent of various mixtures of amino acids in the Osborne and Mendel ration have indicated the inability of such rations to maintain mice at constant weight. Whether this is due to a qualitative insufficiency of the amino acid mixtures or to such a small consumption of the amino acid rations that their adequacy has not been put to a fair test, cannot be said. However, the food consumption in these experiments was subnormal in all cases, and the possibility that therefore the energy requirements were not covered must be conceded a fair probability.

6. More success resulted from the feeding of rations containing from 2.5 to 9 percent of amino acids, alternately with rations containing no protein or amino acids except as these occur in "protein-free milk" or the modified product that has

been described above. On these rations continuous decline in body weight resulted in all cases, though this decline was much more gradual than with the first series of amino acid rations. No evidence of digestive trouble was noticed in this second series of experiments, and symptoms of malnutrition other than the decline in body weight have been deferred for two or three, and in some cases four weeks. No particular difficulty has been experienced in keeping mice alive for 40 or 50 days on this dietary regime.

7. An attempt to determine the relative efficiency of the different amino acid mixtures used in the second series of experiments from data on the percentage losses in weight and the daily intake of total food and of amino acids per 100 grams of body weight, have indicated no certain differences, in spite of the fact that the amino acid mixtures differed markedly in character. The most complete amino acid mixtures used showed to a disadvantage as compared with other mixtures, and, in fact, some of the best results were obtained with mixtures lacking in one or more of those amino acids that have been proven to be physiologically indispensable by other methods of attack. The loss of weight on some of these rations was comparable to the loss of weight of mice fed the Osborne and Mendel ration containing 18 percent of the chemically inadequate protein, zein. The loss of weight with other of our rations was even less than has been obtained with zein.

8. The possibility still exists that the amino acid mixtures used in the second series of experiments were qualitatively adequate, but were not consumed in sufficient quantities. In the alternate feeding of amino-acid rations and non-amino-acid rations, the latter were eaten in greater quantities than the former in all cases.

9. The results obtained in the second series of feeding experiments with amino acid rations were clearly better than those obtained from the feeding of the non-amino-acid ration with a nitrogen content limited to that of the "protein-free milk" contained in them, when this ration was fed continuously. Furthermore, everything considered, they compared favorably with the results obtained from the feed-

ing of rations containing 4 percent of casein supplemented by cystine and tryptophane.

2. Suggestions for Future Work

While the line of investigation initiated by the experiments reported above might conceivably be continued for a long time before positive results are obtained, and might be pursued for many years before anything approximating a complete solution is forthcoming the importance of the problem, and the theoretical and practical value of the results to be secured certainly justify the time, labor, and money involved. It is probably the only method by which the amino acid requirements may be completely discovered. Furthermore, the knowledge of the significance of each amino acid in nutrition will greatly enhance the value of all analytical data on the amino-acid content of proteins and of feedingstuffs. In fact with the meagre information available at the present writing of amino acid requirements for maintenance or growth, the data obtained by such analytical work will have but little practical value or utility.

As stated above, the negative results obtained in this investigation may very well be explained by the small consumption of the amino acid rations. It is quite conceivable that many of the rations tested may have been qualitatively sufficient, but that the mice did not consume enough of them to quantitatively cover their requirements. Further experimentation should therefore first concern itself with this phase of the question, and the feeding of the amino acid mixtures already used should not be discontinued until a reasonable certainty has been established that the amino acid intake has been quantitatively sufficient. A suggestion that might well be tried, if further difficulty is encountered in securing adequate consumption of the amino acid rations, is to feed the amino acids in the form of their sodium or ammonium salts.

If it can be demonstrated that a mixture of all the amino acids ordinarily found in proteins is qualitatively inadequate for maintenance, a logical continuation of the work would be a resort to the Fischer esterification method for the

separation of the amino acids occurring in casein after hydrolysis with enzymes. A determination of which of the distillation fractions obtained by the Fischer procedure contains the amino acids, besides those isolated and tested previously, that are indispensable will indicate the direction for further research.

Once an amino acid mixture is found that will be adequate for maintenance for indefinite periods, further progress in determining the amino acid requirements will envolve straight-forward and systematic work. By witndrawing one after the other of the amino acids contained in such a mixture, the indispensability or otherwise of each may be definitely determined, and by systematically varying the proportions in the resulting mixture of each of those amino thus found to be indispensable, the minimum requirement for that amino acid may be ascertained.

The question of the advisability of the use of "protein-free milk" in experimental work of the character illustrated by this investigation has recently been raised by McCollum and Davis.[1] After referring to the fact that it has been demon-

[1] Journ. Biol. Chem., 20, pp. 646-648, April 1915

strated that a ration containing 3 percent of milk proteins is sufficient for the maintenance of rats, they say:

"There is contained in all the rations fed by Osborne and Mendel, therefore, at least 41 percent of the amount of milk nitrogen necessary to maintain a young rat in body weight, provided the nitrogen is in the form of protein or cleavage products of milk protein which are its equivalent. It is a well-known fact that proteolytic ferments are present in milk, and this fact alone should make us cautious about accepting the results of precipitating 'protein' from milk as an index to the nutritive value of the part of the nitrogen not precipitated by protein reagents. It is true that qualitatively nearly all the constituents of blood have been identified in milk, but these are present in extremely small amounts".

It should be pointed out in this connection that the protein precipitants used by Osborne and Mendel in their analysis of "protein-free milk" (see page 19) are also precipitants for many of the intermediary products of protein hydrolysis, and that these intermediary products are therefore included in the protein values assigned by these investigators to their "protein-free milk". McCollum and Davis have attempted a determination of the actual nutritive value of the nitrogen of

"protein-free milk", with the results described in the following quotation:

"That the nitrogen of protein-free milk is nearly, if not quite, equivalent to milk protein nitrogen as a nutrient for young rats is made evident from experiments which are at present in progress in our laboratory, in which young rats have maintained their body weight during eighty days on a mixture of milk powder protein 15 percent, protein-free milk protein (N x 6.25) 1.5 percent, with dextrin and butterfat to make 100. We have already mentioned that milk protein in the form of skim milk powder fed at levels of 2 percent of the food mixture causes steady loss of weight, while 3 percent of the same proteins serves to maintain body weight in rats for periods beyond 100 days. It is evident that the total nitrogen of protein-free milk serves as nearly, if not quite, the equivalent of so much milk powder nitrogen in such maintenance rations."

Until the results of the work mentioned in the above paragraph are published, no detailed criticism of them can be made. It may be pointed out, however, that McCollum and Davis are very much in error if they assume that the minimum of protein on any ration can be determined merely from the smallest percentage of protein compatible with maintenance of body weight. A determination of the actual intake of protein is absolutely necessary, and in all of their work no determination of food intake seems to have been made by these investigators. The results of their experiment as stated in their own words, have no unequivocal significance, for the simple reason that the food intake upon this ration may have been considerably greater than that upon the rations containing 3 percent of milk proteins. This is easily conceivable since butterfat was added to the former ration and was not included in the latter.

The caution with which results on the comparative efficiency of different levels of protein intake must be interpreted is well illustrated by some of the data of McCollum and Davis given in a previous publication.[1] In testing the ef-

[1] Journ. Biol. Chem., 20, p. 418. March 1915.

ficiency of a ration containing 2 percent of milk proteins, a very gradual decline in weight was generally obtained; in one case, however, an abrupt decrease occurred, and in one case maintenance was secured as long as the experiment was continued -- 49 days.

From such considerations it cannot be concluded that the argument of McCollum and Davis against the use of protein-free milk is valid. While some objection

is involved in the use of this product, we have reduced its force by substituting for the original preparation, a modified "protein-free milk" with a demonstrably lower protein content. Other improvements in the method of preparation of this product are being attempted with the hope of still further reducing its nitrogen content.

If the success of McCollum and Davis with artificial salt mixtures can be duplicated with mice, "protein-free milk" may be entirely dispensed with. Their most successful salt mixture in experiments on mice, however, has been shown to be inadequate in this investigation. Until a successful salt mixture for mice is elaborated, experimentation with "protein-free milk" rations must continue, and the results of such experimentation need not be discounted appreciably by the inclusion of this product of the rations

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In conclusion I take pleasure in acknowledging the general supervision of this work by Professor H. S. Grindley, and the aid which his suggestions have contributed. The assistance of Mr. Anton Prasil and Mr. R. A. Nelson in the laborious work of preparing and synthesizing the amino acids used, is also gratefully acknowledged. The interest of Mr. C. G. MacArthur in this investigation, and the suggestions and encouragement which he has continually given have been highly appreciated. I also take great pleasure in acknowledging the valuable advice and assistance of Professor C. G. Derick and Dr. L. Thorp in the synthetic preparation work.

APPENDIX

1. Composition of Amino Acid Mixtures

2. Body Weight Charts

COMPOSITION OF AMINO ACID MIXTURE

Expressed as grams of amino acids per 100 grams of ration

Amino Acids	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Glycocoll	1.0	1.0	1.0	1.0	0.0	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.50
Alanine	2.0	1.0	1.7	2.0	0.0	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.50
Valine	0.5	1.0	1.0	1.0	0.0	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.50
Leucine	1.5	1.3	1.3	1.3	0.0	0.50	0.25	0.50	0.50	0.50	0.50	0.75	1.00
Isoleucine	0.5	0.7	0.0	0.7	0.0	0.25	0.00	0.00	0.25	0.00	0.00	0.00	0.25
Phenylalanine	1.0	1.0	1.0	1.0	0.0	0.25	0.00	0.25	0.25	0.25	0.50	0.00	0.50
Tyrosine	1.0	1.0	1.0	1.0	1.0	0.50	0.50	0.50	0.50	0.50	0.00	0.00	0.75
Cystine	0.5	0.8	0.8	0.8	1.0	0.25	0.25	0.25	0.37	0.25	0.25	0.25	0.50
Serine	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	0.38	0.00	0.00	0.00	0.00
Proline	1.0	1.0	1.0	0.0	0.0	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.50
Aspartic acid	2.0	1.5	1.5	1.5	0.0	0.25	0.00	0.00	0.25	0.00	0.00	0.00	0.50
Asparagine	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.50	0.45	0.50	0.50	0.75	0.50
Glutamic acid	4.0	4.0	4.0	4.0	4.0	1.00	0.50	0.75	0.75	1.05	1.00	1.00	1.50
Tryptophane	0.5	0.6	0.6	0.6	0.6	0.25	0.25	0.25	0.30	0.00	0.30	0.30	0.30
Arginine	1.0	1.0	1.0	1.0	1.0	0.25	0.25	0.50	0.40	0.45	0.45	0.45	0.50
Histidine	0.5	0.6	0.6	0.6	0.5	0.25	0.25	0.25	0.20	0.25	0.25	0.25	0.30
Lysine	1.0	1.5	1.5	1.5	0.0	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.40
Total	18.0	18.0	18.0	18.0	6.1	4.00	2.50	4.00	6.00	4.00	4.00	4.00	9.00

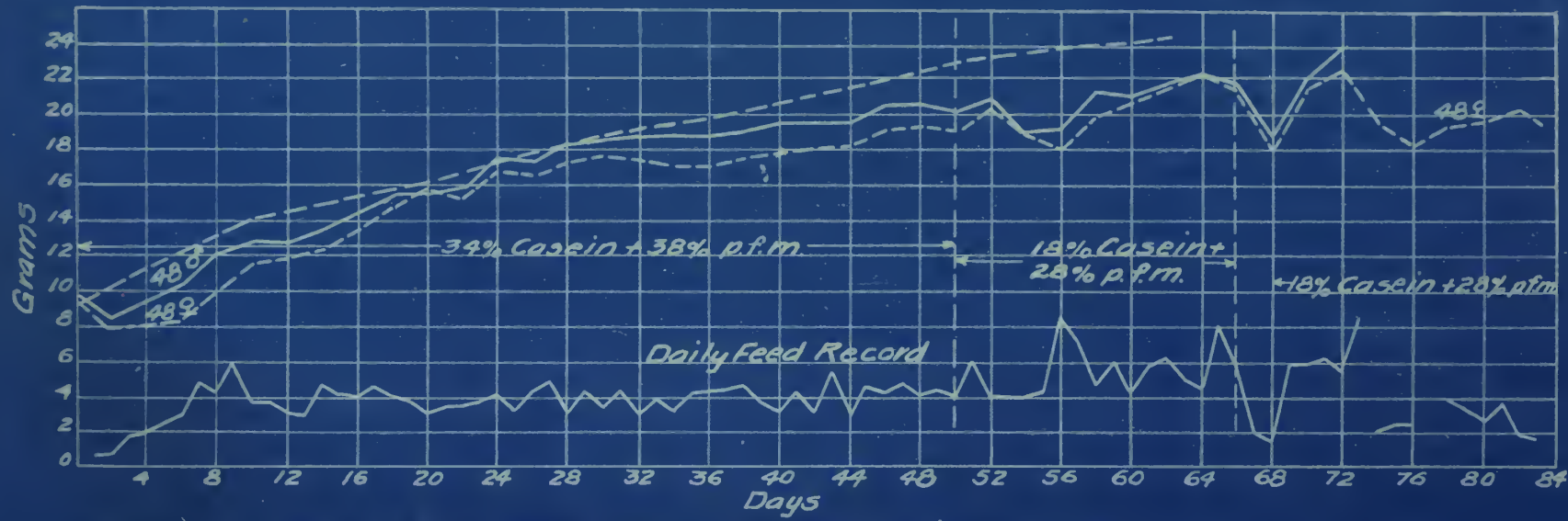

CHART II Two mice from same litter - 28 days old at beginning of test.

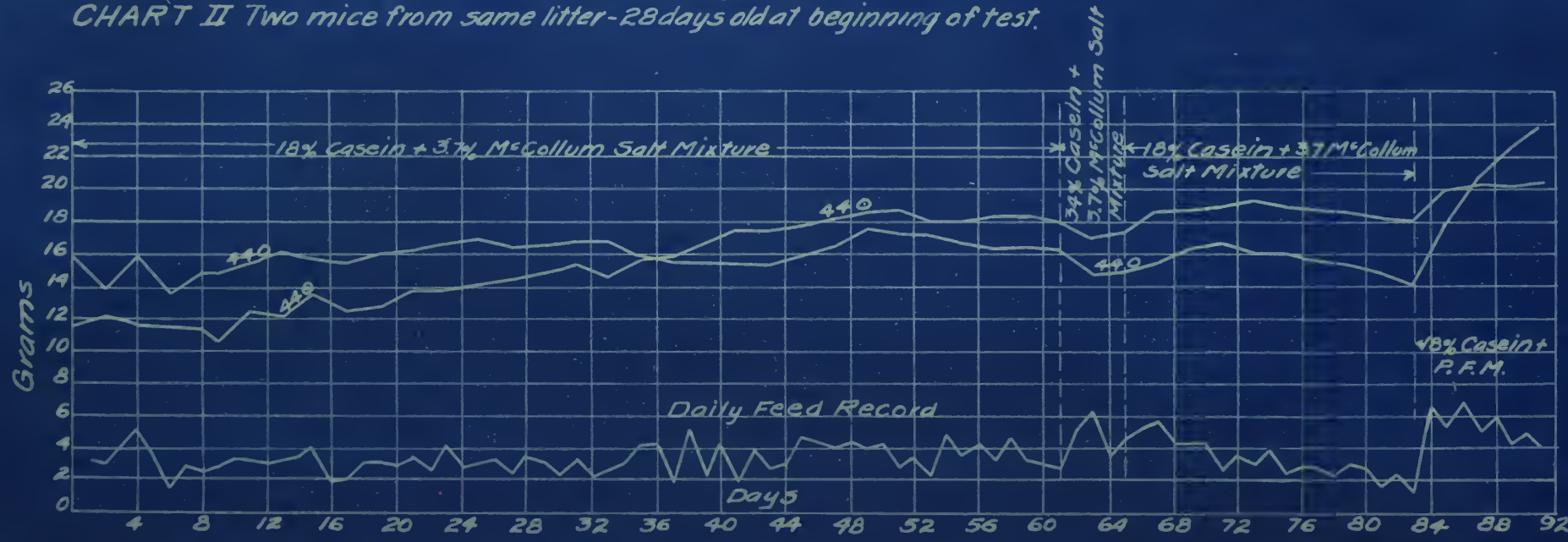

CHART-I Two males from the same litter - 53 days old at beginning of test.
The protein free milk used in the last ration was filtered after neutralization

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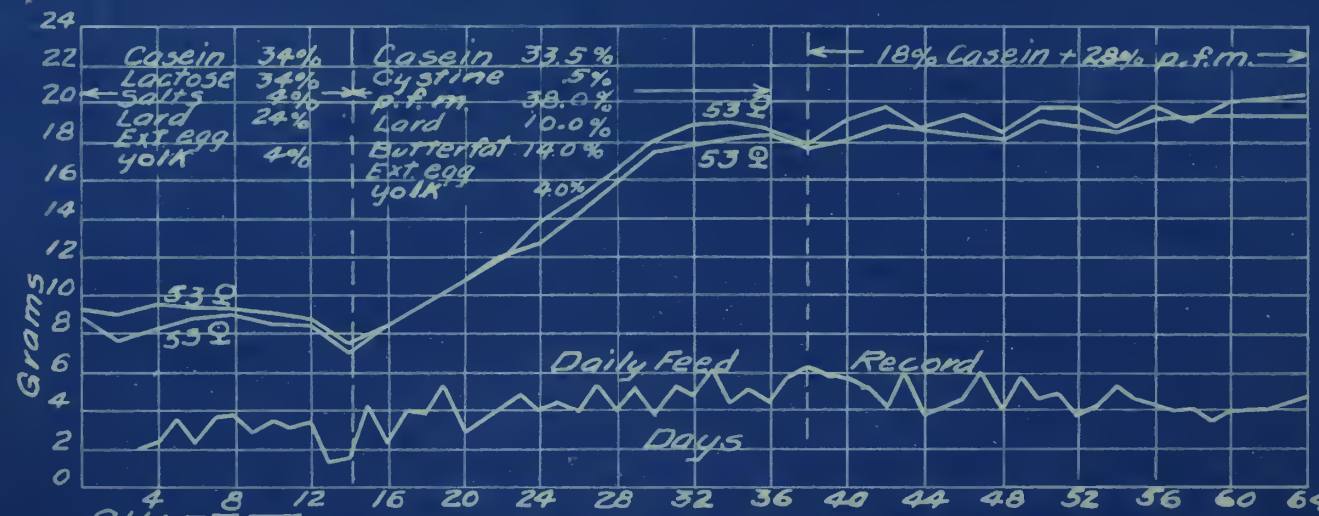


CHART III Two mice from the same litter - 30 days old at beginning of the test.

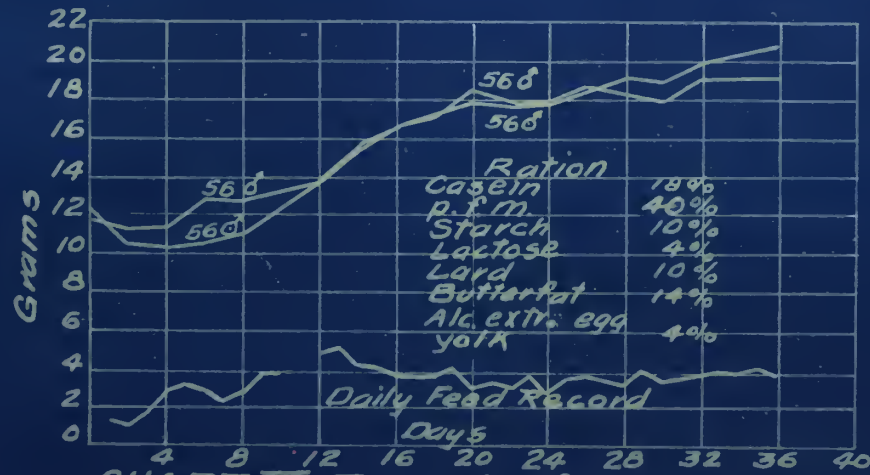


CHART IV Two mice from the same litter. 30 days old at beginning of test.

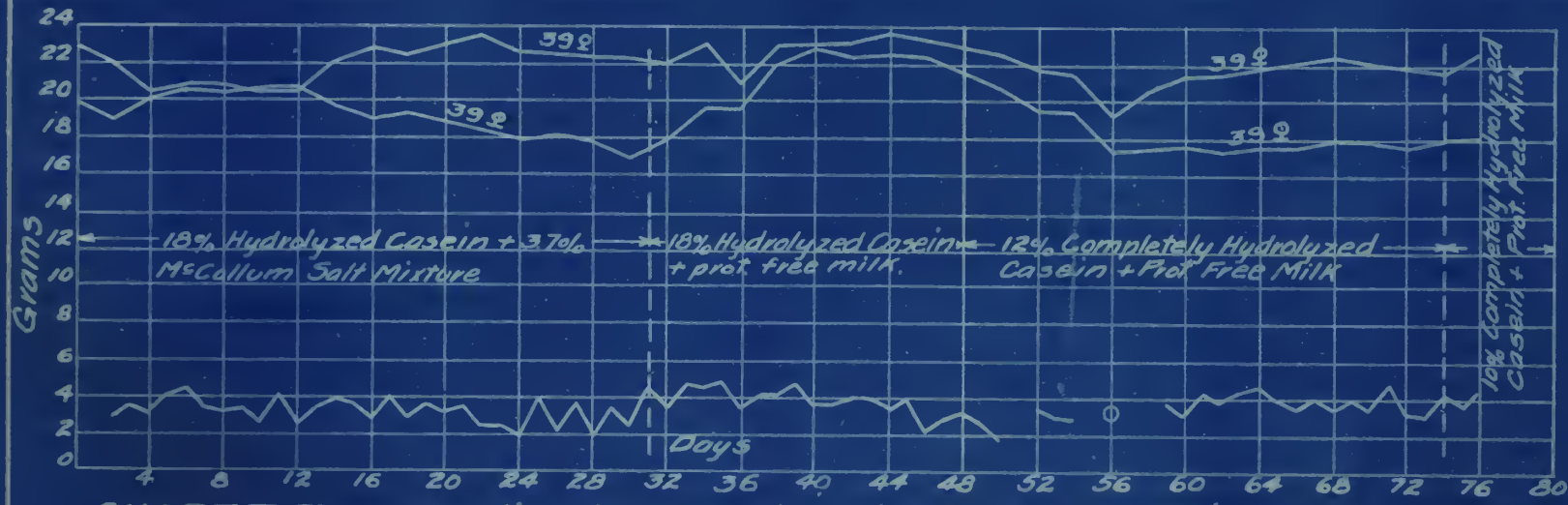
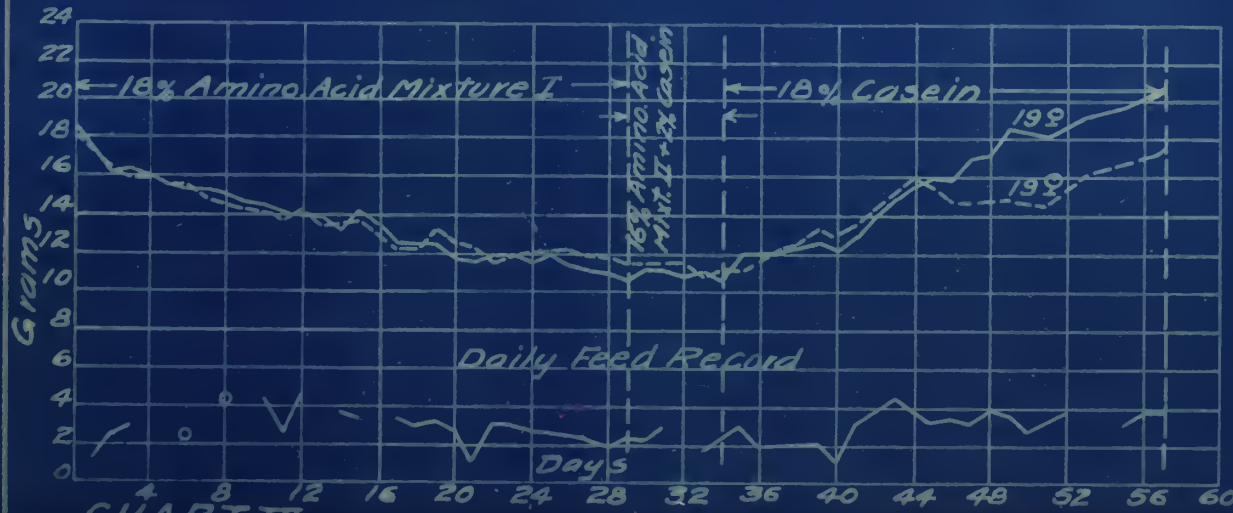


CHART V The preparation of hydrolyzed casein used in the first two rations contained traces of proteoses and peptones



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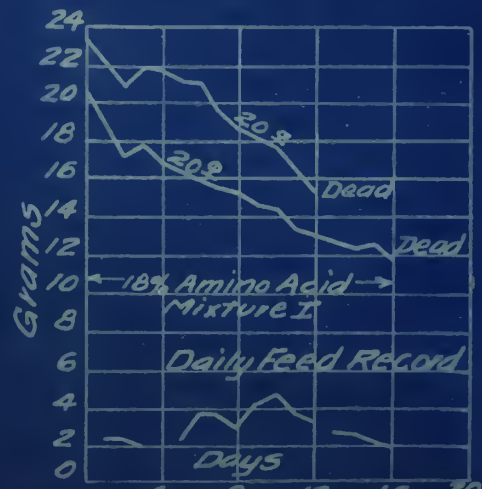

CHART VII - 20♀ died with

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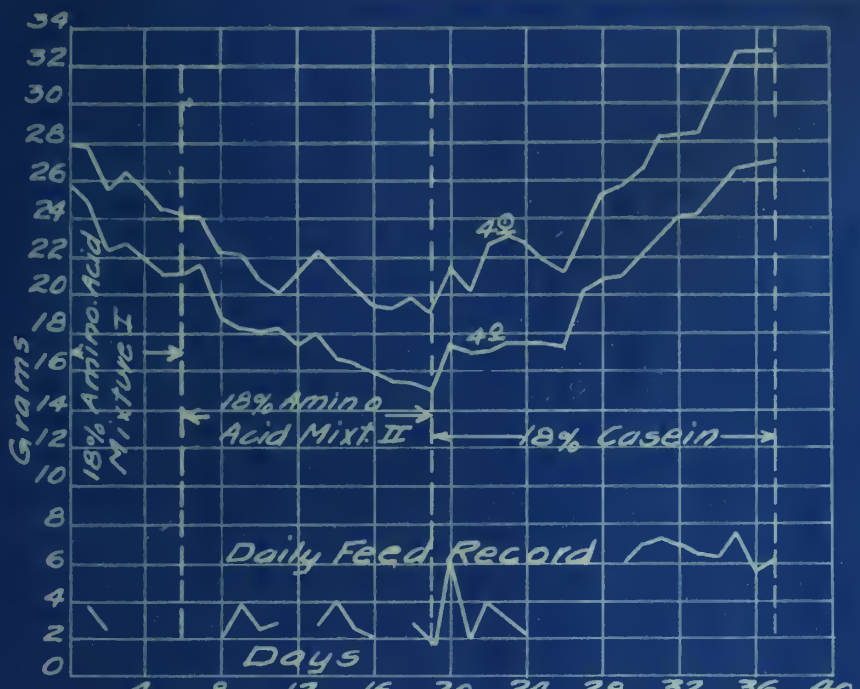

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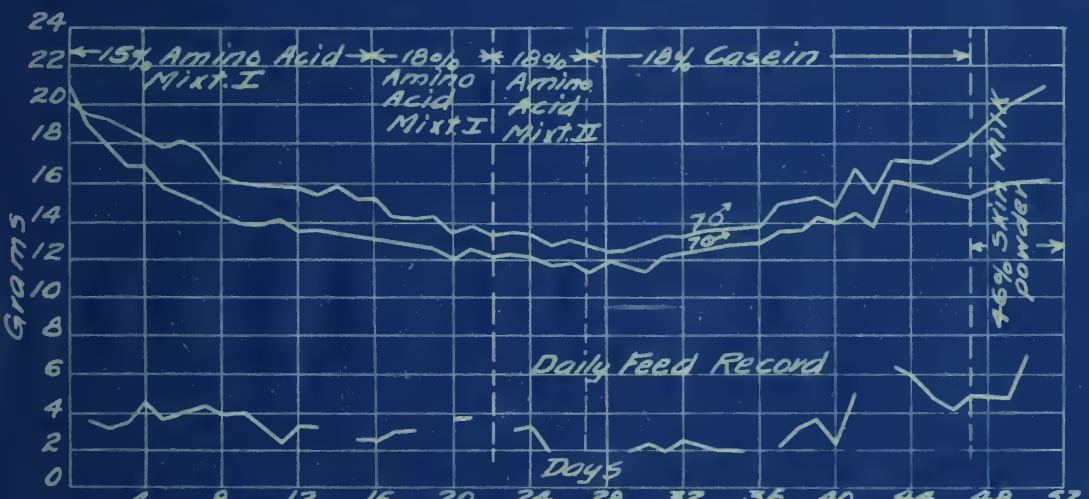

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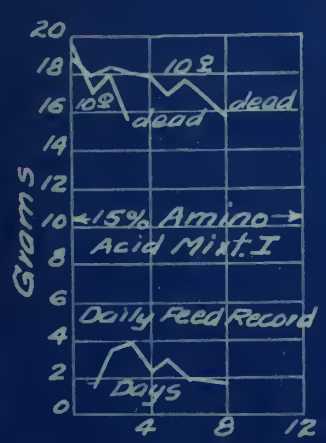

CHART X

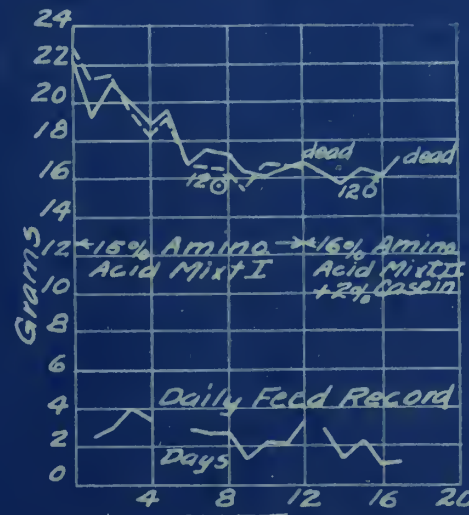

CHART XI – Both of these mice died with symptoms of severe digestive disorder.

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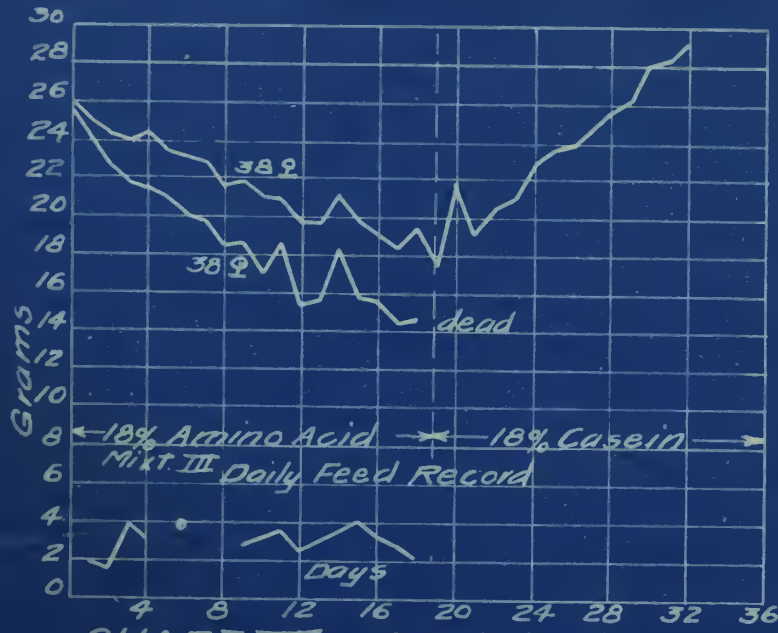

CHART XII - 38♀ Died with symptoms of severe digestive disorder.

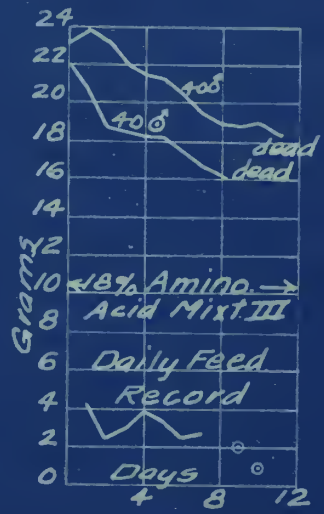

CHART XIII

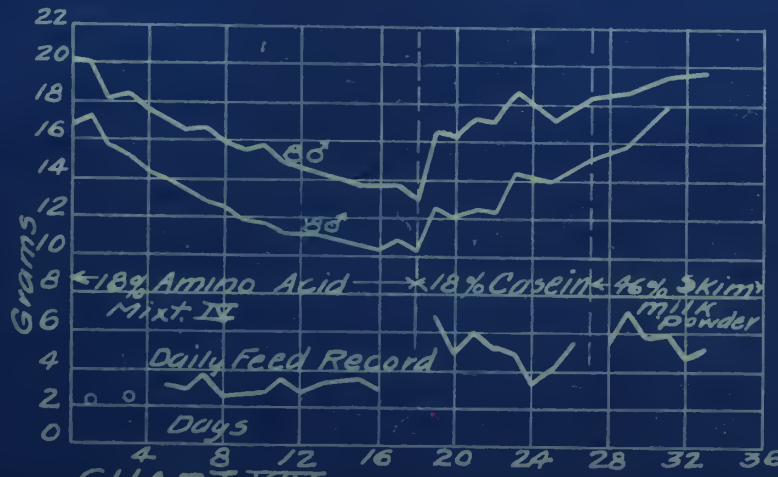

CHART XIV

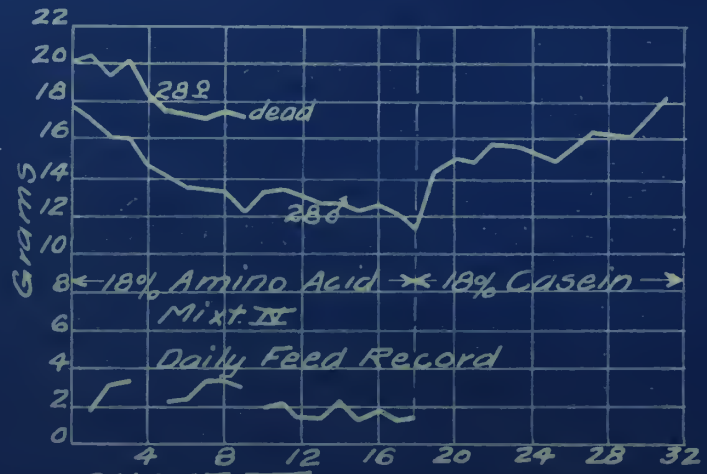

CHART XV

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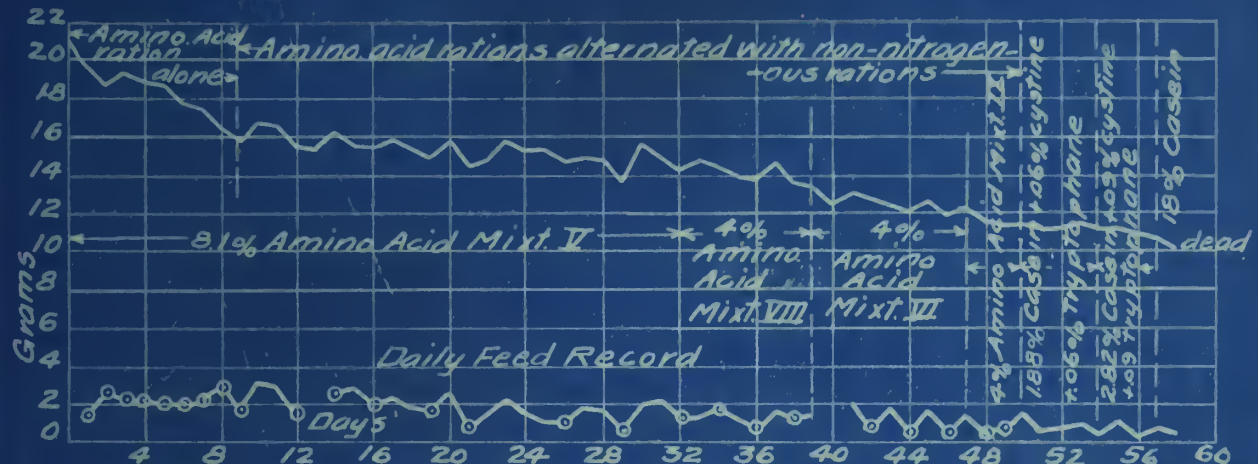

CHART XVII The circled points on the daily feed record indicates the days on which Amino. acid rations were fed. On the 57th day a ration was given containing 2.82% Casein +.09% cystine +.09% tryptophane.

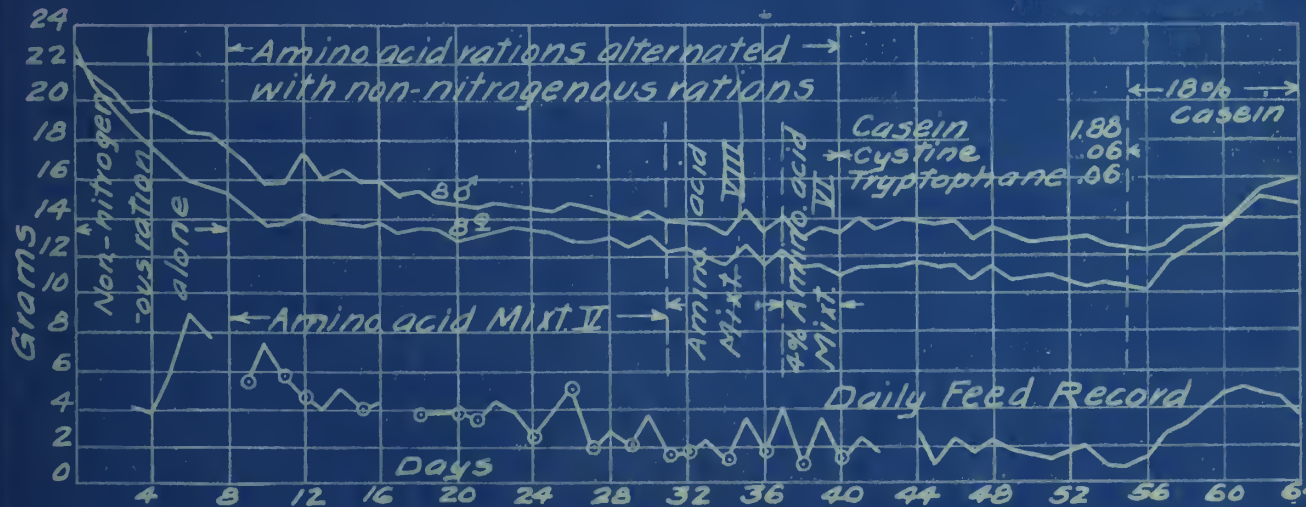

CHART XVIII The circled points on the daily feed record indicate days on which Amino. Acid rations were fed. On the 56th day a ration was given containing 3.76% Casein +.12% Cystine + .12% tryptophane.

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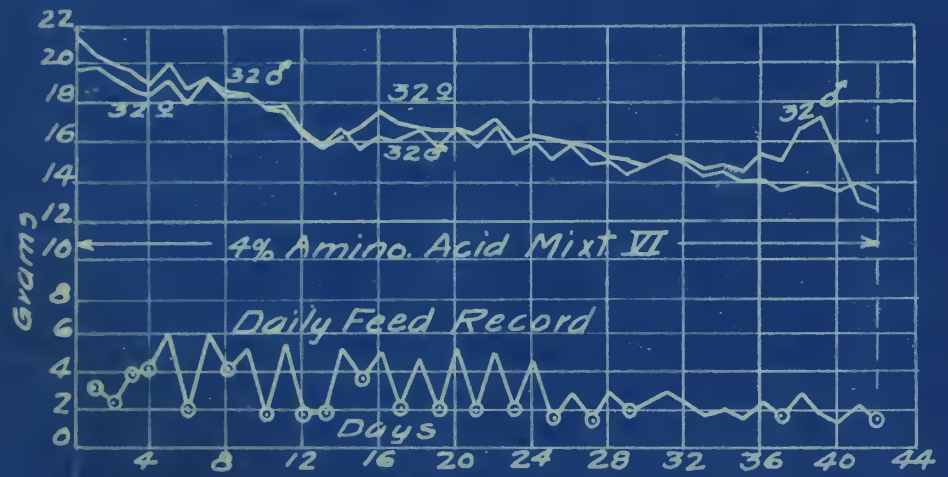

CHART XVIII

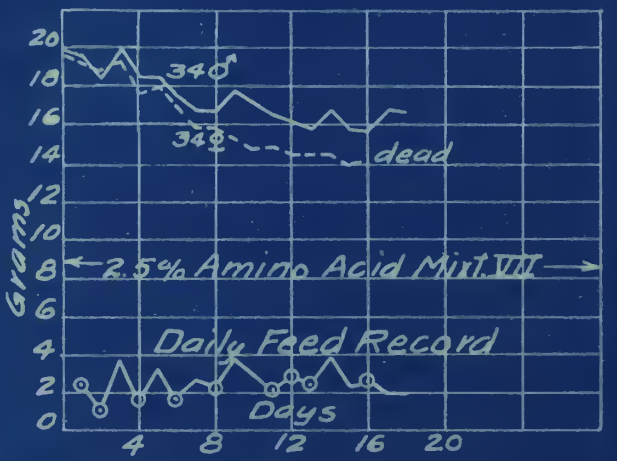

CHART XX

The circled points on the daily feed record curves indicate days on which Amino. Acid rations were fed.

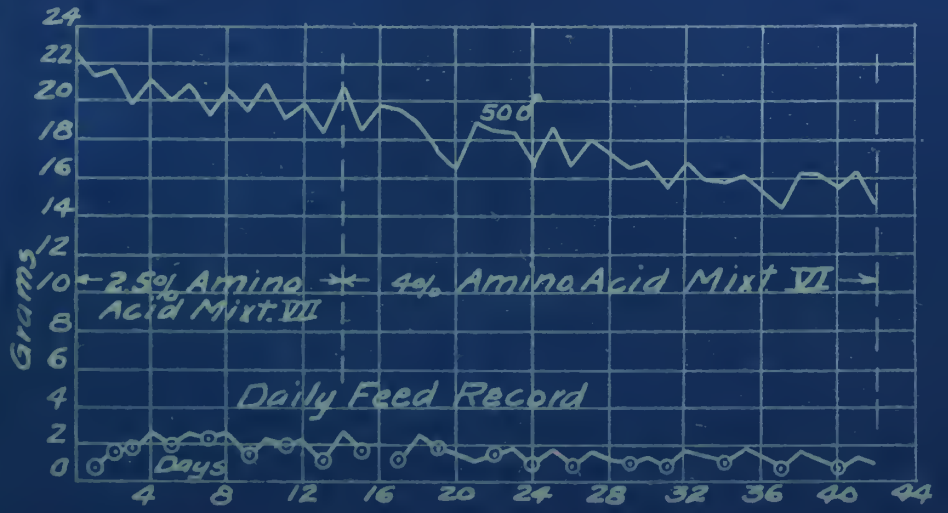

CHART XIX

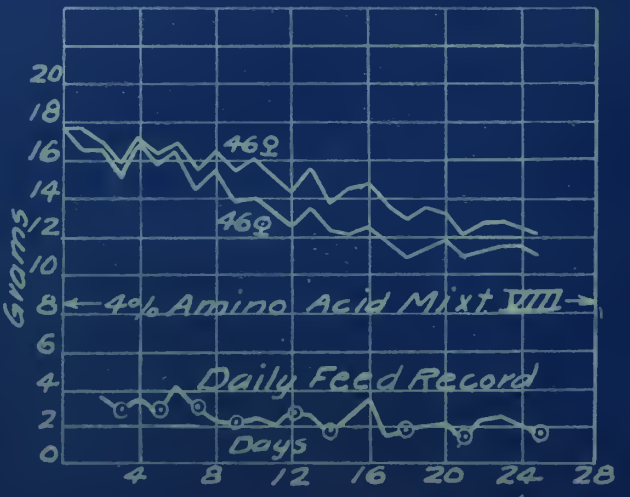

CHART XXI

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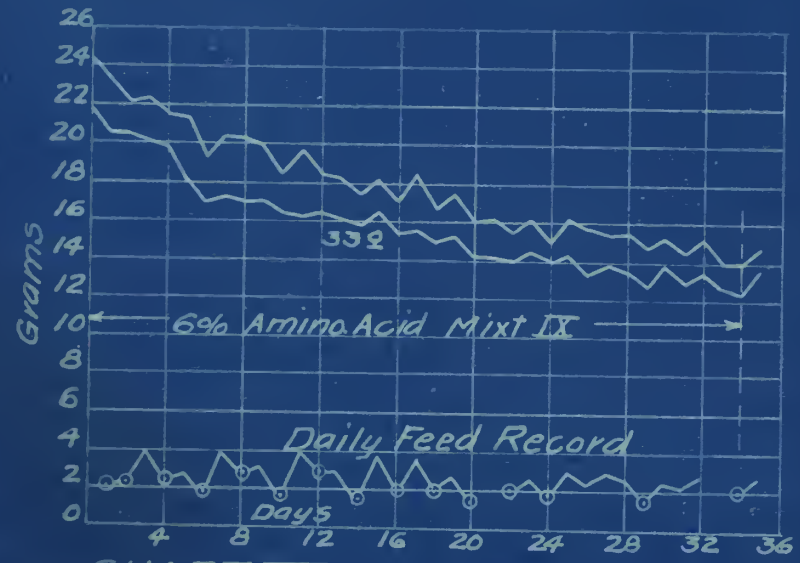

CHART XXII

CHART XXIV

The circled points on the daily feed record curves indicate days on which Amino Acid rations were fed.

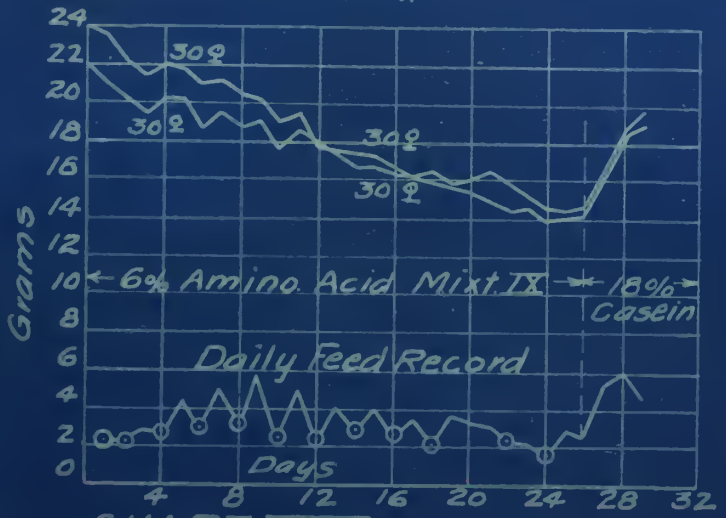

CHART XXIII

CHART XXV

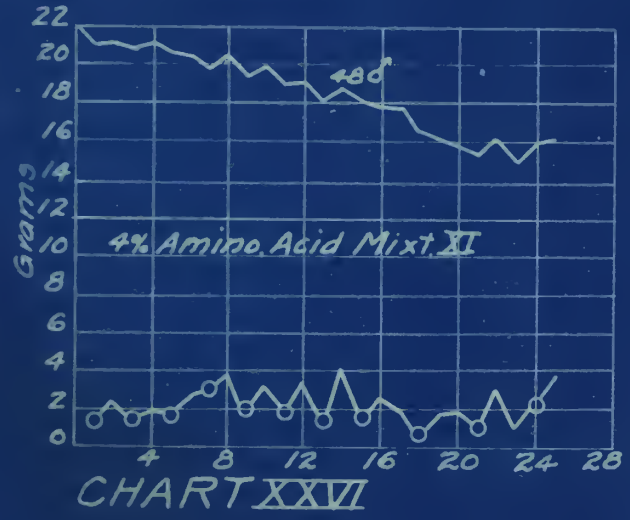

CHART XXVI

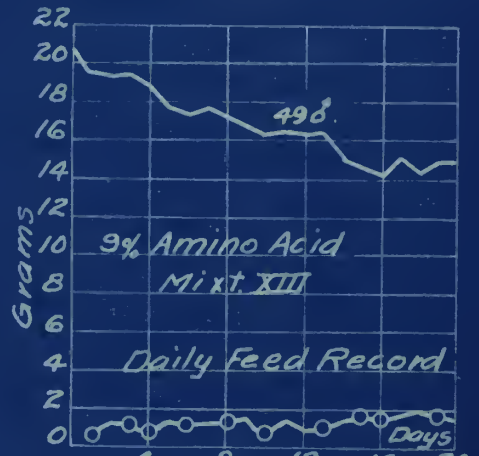

CHART XXVIII

The circled points on the daily feed record curves indicate days on which Amino Acid rations were fed.

CHART XXVII

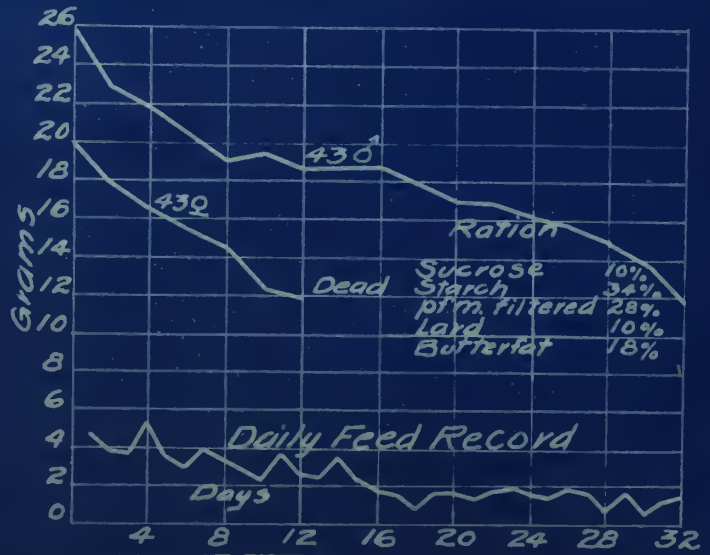

CHART XXIX

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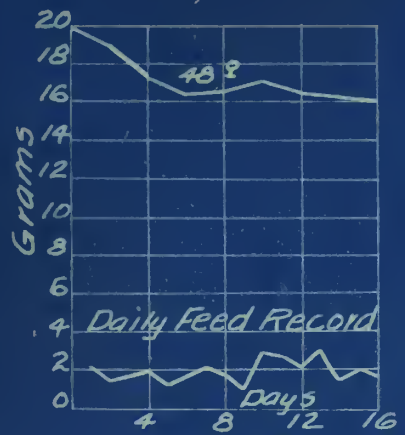

CHART XXX

Same ration as in chart XXIX

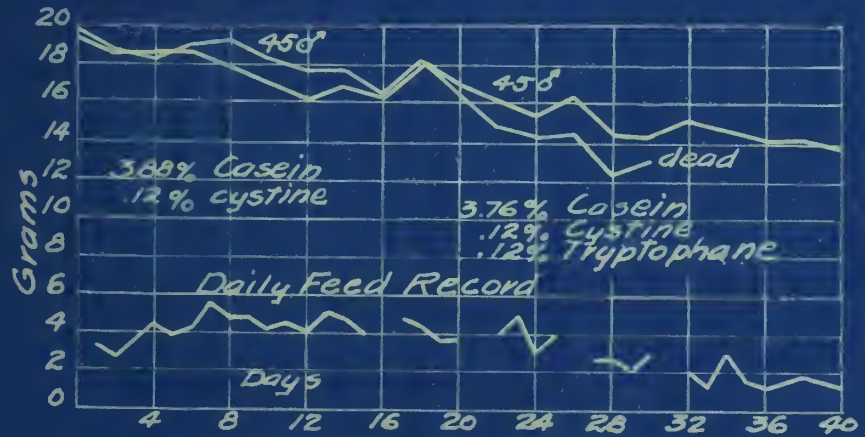

CHART XXXI

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BIOGRAPHICAL SKETCH

The writer was born at Evanston, Illinois, January 22, 1886, and received his elementary education in the public schools of this city and of Chicago, Illinois. After three and a half years attendance at the Hyde Park and the Austin High Schools of Chicago, he entered the University of Illinois in the fall of 1904. He received the degree of Bachelor of Arts in the general science course in June, 1909. After three years in the graduate school of the University of Illinois he received the degree of Master of Science in Chemistry in 1913.

From 1911 to 1913 he held the position of assistant in Chemistry in the Agricultural Experiment Station of the University of Illinois, and since the latter date, he has been an associate in Chemistry in the Agricultural Experiment Station. For the last two years he has done instructional work in animal nutrition.

The writer has published the following papers:

1. A Study of the Food Requirements of a Group of Twenty-Four Men (with H. S. Grindley). Presented before the American Chemical Society, July 1, 1909. Abstract published in Science, Vol. 30, p. 241. 1909.

2. On the Metabolism Experiment as a Statistical Problem (with H. L. Rietz), Journal of Biological Chemistry, Vol. 8, pp. 297-326. October 1910.

3. The Element of Uncertainty in the Interpretation of Feeding Experiments (with H. S. Grindley). Illinois Agricultural Experiment Station, Bulletin 165. 117 pp. July 1913.

4. Does a Low Protein Diet Produce Racial Inferiority? Science, Vol. 38, pp. 82-83. 1913.

The writer is a member of Sigma Xi and Phi Lambda Upsilon.